University of Alberta

Mechanisms of Tumor-Cell Induced Platelet Aggregation

by

Paul Konrad Jurasz

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

Department of Pharmacology

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Abstract

Tumor-platelet aggregates play an important role in tumor spread. The ability of tumor cells to aggregate platelets, i.e. tumor cell induced platelet aggregation (TCIPA), confers a number of advantages to the survival of tumor cells in the vasculature and to their successful metastasis. Understanding the mechanisms of TCIPA may lead to novel inhibitors of hematogenous metastasis. Matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play crucial roles in metastasis and platelet aggregation. Additionally, TCIPA and the arrest of tumor containing aggregates depend on platelet surface receptors. Therefore, I have studied the roles of MMP-2 and TIMP-4 in TCIPA using human HT-1080 fibrosarcoma and A549 lung carcinoma cells to induce TCIPA. Furthermore, I have investigated the role of two major platelet receptors: glycoprotein Ib (GPIb) and IIb/IIIa (GPIIb/IIIa) in TCIPA using a novel solid phase agonist, von Willebrand Factor (vWF) immobilized on polystyrene beads. In addition, the role of nitric oxide (NO) and prostacyclin (PGI_2) in regulating TCIPA was also studied. It is concluded that (1) human tumor cells aggregate platelets via mechanism(s) that are mediated, in part, by MMP-2, (2) NO inhibits TCIPA by inhibiting the release of MMP-2, (3) these effects of NO are cGMP-dependant, (4) TCIPA results in the expression of platelet GPIIb/IIIa, while decreasing GPIb expression, (5) vWF potentiates TCIPA, (6) these effects of vWF are inhibited by NO and PGI_2, (7) NO and PGI_2 differentially affect the expression and activation of GP IIb/IIIa, (8) platelet TIMP-4 is not released during TCIPA, and (9) pharmacological recombinant TIMP-4 has the ability to modulate TCIPA.

Dedication

I would like to dedicate this thesis to my parents Maria and Tadeusz Jurasz. Their support throughout my PhD studies, encompassing the last four years, and my entire academic career has been tremendous. I would like to thank them for their unyielding support and trust in my dreams, decisions, and actions. I would like to thank them for their patience, understanding, and guidance over the last four years. Thanks mom and dad.

Furthermore, I'd like to also dedicate this work to my brother Martin and his wife Antoinette. They have been wonderful supporters of mine with whom I could share my successes and failures. Thanks for listening.

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List of Abbreviations

a/bFGF	acidic/basic Fibroblastic growth factor
AC	Adenylyl cyclase
ADP	Adenosine diphosphate
APC	Activated protein C
β-TG	β-thromboglobulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
CTAP-III	Connective tissue activating peptide
DTS	Dense tubular system
EDRF	Endothelium-derived relaxing factor
EGF	Epidermal growth factor
FITC	Fluorescein isothiocyanate
GP Ib	Glycoprotein Ib
GP IIb/IIIa	Glycoprotein IIb/IIIa
GC-S	Soluble guanylyl cyclase
GSNO	S-nitrosoglutathione
IL-1	Interleukin-1
IL-8	Interleukin-8
MCP-1	Macrophage chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteinase

NAP-2	Neutrophil-activating peptide 2
NO	Nitric oxide
NOS	Nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
PDE	Phosphodiesterase
PF-4	Platelet factor 4
PG	Prostaglandin
PGI_2	Prostacyclin
PKA	Protein kinase A
PKG	Protein kinase G
$O_2^{\cdot-}$	Superoxide
OCS	Open canalicular system
ODQ	1H-[1,2,4]Oxadiazole[4,3]quinoxalin-1-one
$ONOO^-$	Peroxynitrite
RANTES	Regulated on activation normal T cell expressed and secreted
RPE	R-phycoerythrin
SNAP	S-nitroso-acetyl-penicillamine
sVWF	Solid phase-von Willebrand Factor
TCIPA	Tumor cell-induced platelet aggregation
THB_4	Tetrahydrobiopterin
TIMP	Tissue inhibitor of matrix metalloproteinases

TNF-α	Tumor necrosis factor α
TPA	Tissue plasminogen activator
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
vWF	von Willebrand Factor

Introduction

Hemostasis

The primary function of the hemostatic system is the prevention of blood loss when a blood vessel is injured. This is accomplished by three very important parts of the hemostatic system: platelets, the coagulation cascade, and the fibrinolytic system. Platelets form the plug that seals the rupture in the blood vessel, the coagulation cascade results in the generation of a fibrin mesh that strengthens the platelet plug, and finally the fibrinolytic system remodels this plug. One of the most common consequences of cancer is the abnormal reactions of the haemostatic system, which was the motivation for this study. While the focus of this thesis is on the interactions of tumor cells with platelets, a general overview of the coagulation and fibrinolytic systems will be provided first.

Coagulation and Fibrinolytic Systems in Health and Cancer Disease

The principle role of the coagulation and fibrinolytic systems is to maintain hemostasis. When a blood vessel is cut or damaged a series of enzymatic reactions lead to the formation of a clot that arrests hemorrhage. Initially, a platelet plug is formed to prevent further blood loss. This platelet plug is then strengthened by the deposition of fibrin and the formation of a clot. The basic mechanism of clot formation, and the last step in the coagulation enzymatic cascade, is the conversion of soluble fibrinogen to insoluble fibrin by thrombin. Two pathways may initiate the enzymatic cascade that leads to coagulation. The first pathway is known as the "extrinsic" or "in vivo" pathway, and the second is

known as the "intrinsic" or "contact" pathway. Classically, the extrinsic pathway was named as such because the components that initiate this pathway were thought to derive from outside of the blood when tissue is damaged. The second pathway was named the intrinsic pathway because all the components that initiate coagulation were thought to be found in the blood. The enzymatic cascade of the intrinsic pathway is initiated by exposure of the blood to a negatively charged surface such as glass or collagen fibers. However, collagen is a subendothelial protein not present in the blood, and comes into contact with the blood only during tissue damage. Therefore, in reality, the two pathways are not separate entities, but for the sake of simplicity they will be described as such.

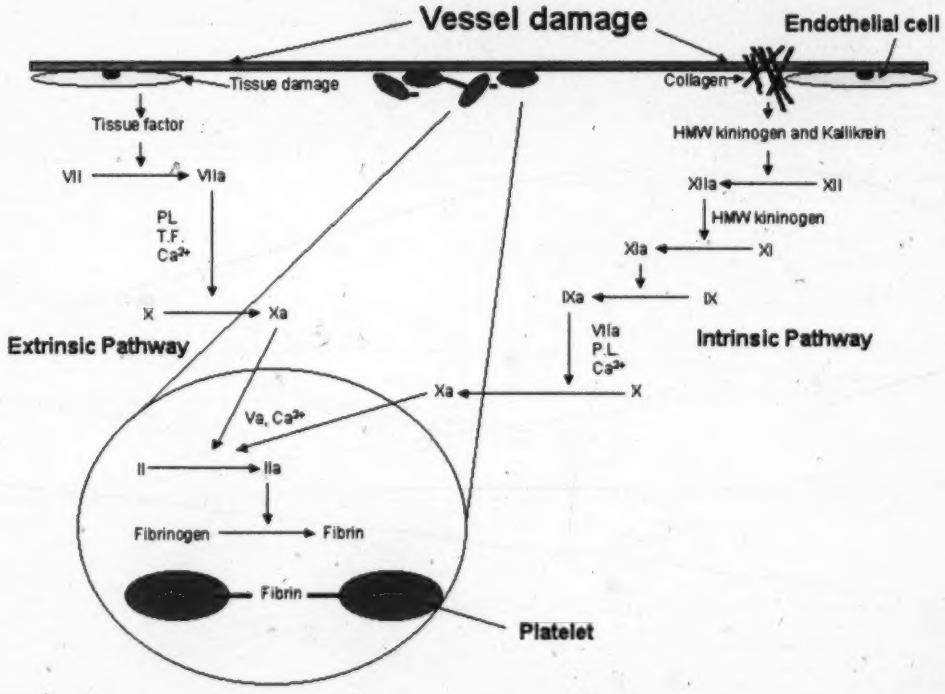

Figure 1. The extrinsic and intrinsic pathways of the coagulation system. PL = phospholipid, T.F. = tissue factor. Please see the text for description of coagulation factors.

Coagulation via the extrinsic pathway is initiated when tissue damage to a blood vessel leads to the release of tissue factor also known as thromboplastin (Figure 1). Tissue factor then activates factor VII that becomes factor VIIa, and along with thromboplastin, Ca^{2+}, and negatively charged phospholipids supplied by activated platelets enzymatically activates factor X (prothrombinase) into factor Xa (thrombinase). In addition, tissue factor and factor VIIa will also activate factor IX of the intrinsic pathway.

The intrinsic pathway is initiated by the exposure of collagen fibers to the blood *in vivo*, or exposure of blood to glass *in vitro*, leading to the activation of high-molecular weight (HMW) kininogen and kallikrein. These two enzymes then activate factor XII into XIIa. Activated factor XII along with HMW kininogen then activates factor XI into XIa. XIa then converts factor IX into IXa, and then factor IXa in the presence of factor VIIIa, Ca^{2+}, and phospholipids converts factor X into Xa. At this point the two pathways converge in the generation of thrombin.

Thrombin is formed from pro-thrombin (factor II) by clotting factor Xa that is also known as prothrombinase. Once again, this reaction requires Ca^{2+}, phospholipids, and activated clotting factor V. Finally, as mentioned above, thrombin cleaves soluble fibrinogen into insoluble fibrin to form the definite clot. This clot is further stabilized by activated factor XIII and Ca^{2+}.

The primary function of the coagulation and fibrinolytic systems is to maintain hemostasis. Therefore, to prevent the uncontrolled formation of blood clots a number of systems exist to prevent progression of physiological hemostasis to pathological thrombosis and to keep the blood in a fluid state. These include the

generation of prostacyclin (PGI_2) by endothelial cells, which inhibits platelet aggregation, and the generation of nitric oxide (NO) by platelets and endothelial cells, which leads to the inhibition of platelet adhesion, aggregation, and dilatation of the blood vessel lumen to maintain blood flow. In addition to the platelet regulator systems, the fibrinolytic system functions to limit and finally to dissipate the hemostatic clots.

The principle active component of the fibrinolytic system is plasmin. Plasmin (fibrinolysin) is an enzyme that proteolytically cleaves fibrin and fibrinogen. It is derived from plasminogen by the action of tissue plasminogen activator (TPA). In addition, this system may be amplified by the binding of thrombomodulin to thrombin. Thrombomodulin is a thrombin-binding protein produced by the endothelium. When it binds to thrombin, an anti-coagulant complex is formed. The thrombomodulin-thrombin complex then activates protein C, which in turn inactivates inhibitors of tissue plasminogen activator allowing for the increased generation of plasmin. In addition, the activated protein C (APC) may also inactivate factor V of the coagulation system. Furthermore, the fibrinolytic system contains anti-thrombin III, an inhibitor of serine proteases that are involved in the activation of the coagulation cascade. Anti-thrombin III inhibits the active forms of coagulation system proteins such as factors IX, X, XI, and XII. The effects of anti-thrombin III are increased by the polysaccharide heparin. Heparin binds to anti-thrombin III and accelerates its inhibitory action on factor Xa and thrombin.

Numerous experimental and clinical studies have shown that in the presence of malignant disease the coagulation system is activated. Metastasizing tumor

cells, both animal and human, have been shown to express a cysteine protease that directly activates factor X and consequently activates the coagulation system (Falanga et al. 1987, Donati et al. 1986). This protease has been given the name "cancer procoagulant (CP)" (Falanga and Gordon 1985). Furthermore, histological examination of ductal adenocarcinoma of the pancreas has shown the presence of tissue factor, an activator of the extrinsic pathway of coagulation, on the surface of cancerous cells (Kakkar et al. 1995). In addition, cancer cells are known to express a pro-thrombinase complex on their surface (Inuyama et al. 1997, Nakata et al. 1998.) Moreover, clinical studies have shown that cancer patients have a hypercoagulable state characterized by increased propensity to arterial and venous thrombosis (Billroth 1878, Peck and Reiquam 1973, Dube et al. 1974). Indeed, thrombosis is the second most common cause of death in cancer patients (Donati 1995), and it has been shown that patients receiving low-dose heparin or warfarin, in combination with chemotherapy, have a greater survival rate than those receiving chemotherapy alone (Clagett and Reisch 1988, Zacharski et al. 1984).

Platelets in the Spread of Cancer: A History

From 19th Century Concepts to Present Day Tumor Cell-Induced Platelet Aggregation

Armand Trousseau

Theodor Billroth

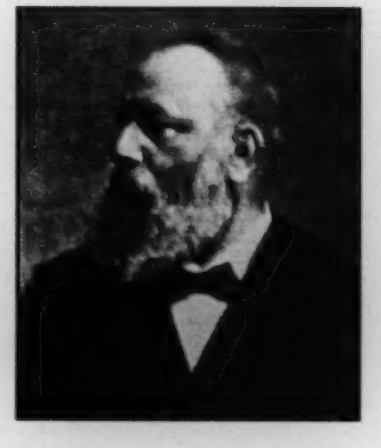

Figure 2. Discoverers of the role of thrombi in metastatic cancer. Sources: www.nlm.nih.gov. and www.medinfo.ufl.edu.

The concept that formation of vascular thrombi can contribute to the development of cancer metastatic disease is not new. Since the late nineteenth century, it has been well documented that thrombi play an integral role in the hematogenous spread of cancerous cells during the metastatic cascade. In 1865, Armand Trousseau reported a high incidence of venous thrombosis in patients with gastric carcinomas (Trousseau 1865). Subsequent work by Theodor Billroth, in 1878, showed that on autopsy human tumor cells are frequently found in association with thrombi (Billroth 1878) (Figure 2). This led Billroth to propose that the hematogenous spread of cancerous cells may be accomplished by tumor cell containing thrombi when he wrote in this treatise:

"If on the first formation of a tumour the lymph vessels should become partially closed and filled with cells, it is quite possible that in the further course of the disease lymph and vascular thrombi should be caused by this stenosis, into which the specific tumour elements may wander, and then minute particles of thrombus may be carried into the circulation first here and then there, and so give rise to further growths. The formation of such thrombi filled with specific tumour elements has actually been observed in larger and smaller veins, and in the branches of the pulmonary arteries."

Since platelet aggregates form one of the main components of such thrombi (Bizzozero 1882) many scientists throughout the latter half of the twentieth century have investigated tumor cell-platelet interactions. **The body of scientific evidence from the last 120 years has undeniably revealed that platelet aggregation by tumor cells plays a critical role in the pathology of metastasis.**

Cancer cells have been shown to have the ability to aggregate platelets (Figure 3) (Gasic et al. 1968 and 1973), and this ability to aggregate platelets correlates with the metastatic potential of the tumor cell (Radomski et al. 1991). The ability of malignant tumor cells to aggregate platelets, i.e. **tumor cell-induced platelet aggregation (TCIPA)** (Gasic et al. 1968 and 1973) confers a number of advantages to the survival of the tumor cell in the vasculature and in its successful metastasis. When covered with a coat of platelets, a tumor cell acquires the ability to evade the body's immune system. Indeed, it has been shown that platelets protect tumors from TNF-α mediated cytotoxicity (Shau et al. 1993, Phillipe et al. 1993). In addition, platelets may shield cancerous cells from

the high shear forces seen in flowing blood that could potentially damage the tumor cell. Another survival advantage for the tumor cell is the tendency for the large tumor-platelet aggregate to embolize the microvasculature at a new extravasation site (Malik et al. 1983). Furthermore, platelets facilitate the adhesion of tumor cells to the vascular endothelium (Mehta 1984), and release a number of growth factors that can be used by tumor cells for growth (Honn et al. 1992). Recently, it has been shown that platelets contribute to tumor-induced angiogenesis by releasing angiogenic growth factors such as vascular endothelial growth factor (VEGF) (Salgado et al. 1999, Salven et al. 1999, Verhuel et al. 2000).

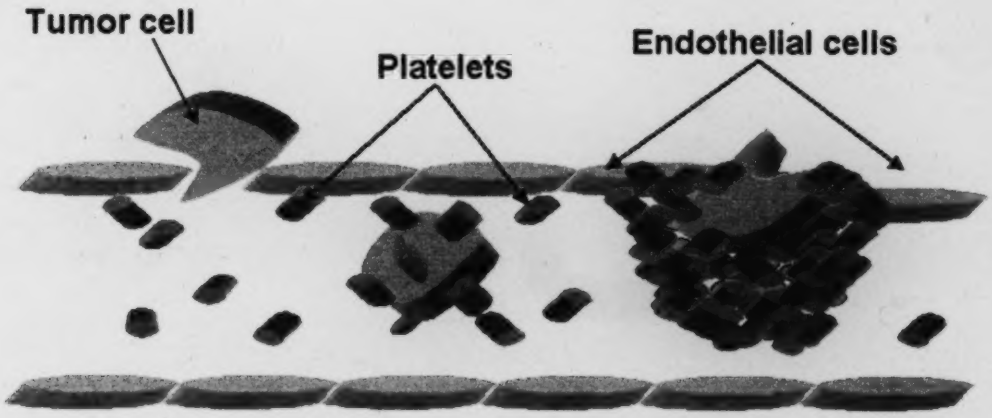

Figure 3. Schematic representation of tumor cell-induced platelet aggregation (TCIPA.)

A number of mechanisms capable of mediating TCIPA have been demonstrated. During TCIPA, caused by certain tumors, there is a release of ADP that stimulates platelet receptors and induces aggregation (Bastida et al. 1982). Other tumors stimulate aggregation and subsequently activate the

coagulation cascade through the generation of thrombin (Bastida et al. 1982, Pearlstein et al. 1981). Furthermore, it has been shown that TCIPA is associated with the production of eicosanoids, such as thromboxane A_2 that amplifies platelet aggregation (Honn et al. 1987). Along with the production of eicosanoids Steinert et al. have shown that platelet surface glycoprotein $\alpha_{II}\beta_3$, the receptor for fibrinogen, plays an important role in platelet aggregation by tumor cells (Steinert et al. 1993). Moreover, Oleksowicz et al. have demonstrated that MCF-7 human breast cancer cells not only express the platelet glycoprotein $\alpha_{II}\beta_3$, but also glycoprotein Ibα, which is the major platelet receptor that binds the subendothelial protein von Willebrand Factor (vWF) and mediates platelet adhesion to the vascular wall (Oleksowicz et al. 1995, Oleksowicz et al. 1997).

Tumor Cell-Induced Platelet Aggregation: The Cellular Players

The Platelet: an Overview

At the same time when Trousseau and Billroth described the role of thrombi in cancer biology, it was discovered that platelets constitute the integral part of thrombi. In 1875, Zahn first reported that the arrest of bleeding occurs as a result of the formation of a white thrombus at the site of a vessel lesion (Zahn 1875). Several years later, in 1882, Giulio Bizzozero reported that the thrombotic mass responsible for hemostasis is formed by the accumulation of platelets (Bizzozero 1882). Thus, Bizzozero is credited with the discovery of the platelet (De Gaetano 2001).

Platelets are anucleate cell fragments derived from the fragmentation of large multinucleate precursor cells known as megakaryocytes (Leven 1987). Once formed from megakaryocytes, platelets have a circulating life span of 10-12 days (Harker and Finch 1969); thus, around ten percent of the platelet population is turned over daily. The platelet is small in size ranging from 2-4 μm in diameter (Klinger 1997). In its inactivated state it is discoid in shape; however, upon exposure to aggregating agents platelets undergo shape change becoming spherical and extending pseudopodia in many directions (Figure 4) (White 1987).

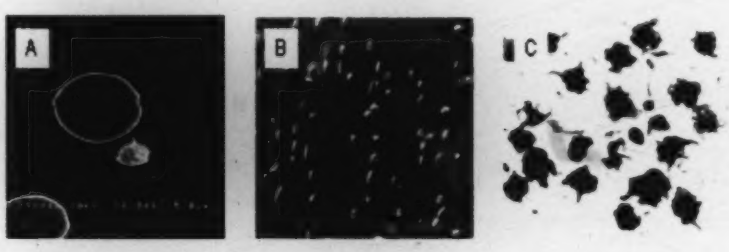

Figure 4. Resting (A and B) and activated platelets (C). Scanning electron microscopy (A, x12,000), phase contrast microscopy (B, x800), and transmission electron microscopy (C, x6,500).

Furthermore, platelets have a number of specific features. One of a platelet's most characteristic features is its lack of a nucleus. Thus, in technical terms, the platelet is not considered as a cell, but as a cell fragment. Because platelets lack a nucleus they are unable to produce substantial amounts of mRNA. However, they do contain some megakaryocyte derived mRNA (Roth et al. 1989), but have very limited translating capacity, thus they are unable for the most part to synthesize new protein (Booyse and Rafelson 1967). The outer surface of the

platelet membrane is rich in glycoproteins, and it is known as the exterior coat or glycocalyx (White 1979). These glycoproteins largely make up the receptors for stimuli triggering platelet activation, adhesion, and aggregation (Nurden and Caen 1974, Nurden and Caen 1975, Philips et al. 1975). Stimulation of these receptors leads to activation of intracellular transduction signaling pathways responsible for mediating platelet reactions, and results in "inside-out" signaling (Levy-Toledano 1999, Fox 1996). Inside-out signaling in platelets describes the process whereby any one of the many platelet agonists that stimulate platelet reactions binds to its surface membrane receptor leading to an activation of intracellular platelet signaling pathways. Platelet stimulation then mediates a change in the conformational state of the glycoprotein IIb/IIIa (GP IIb/IIIa) leading to its activation. This regulation of GP IIb/IIIa may be dependent, in part, on the activation of protein kinase C in platelets. This receptor mediates platelet-platelet aggregation interactions. Furthermore, the platelet surface is covered with integrins (Ginsberg et al. 1995, Goodman et al. 1993, Parise 1989), nucleoside receptors (Puri and Colman 1998), and Fc-receptors that bind antibodies (Parbatani et al. 1987, Shido et al. 1995). On the platelet surface many membrane openings and channels make up the surface-connected open canalicular system (OCS) (Behnke 1970). The precise role of the OCS in platelet physiology has been debated extensively for some time. This stems from the question, "How exactly do platelets mediate the process of secretion, whether through the formation of exocysed vacuoles or through the OCS?" Extensive studies mostly employing electron microscopy show that the OCS is the system,

that provides an exit for products of the platelet release reaction and provides access to the platelet interior for plasma borne substances (Figure 5) (White and Krumwiede 1987, White 2000).

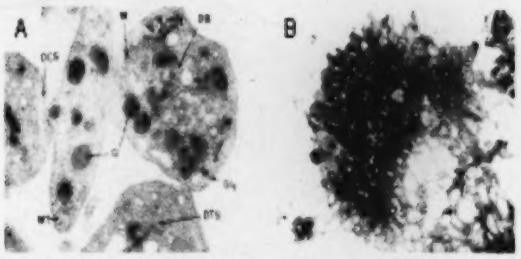

Figure 5. Transmission electron microscopy of platelet organelles (A, x40,000), and the release reaction (B, x40,000) (Open canalicular system, OCS; dense tubular system, DTS; dense bodies DB; microtubules, MT; mitochondria, M; G, alpha-granules; and Glycogen stores, Gly.

The matrix of the platelet is found on the cytoplasmic side of the membrane. This fibrous system contains actin and myofilaments that make up the cytoskeleton that supports the platelet discoid shape and provides the contractile machinery for platelet shape change, pseudopod formation, and secretion (White 1983 and 1984). Another characteristic feature of the platelet is the dense tubular system (DTS) that is often found in association with the OCS (Figure 5) (White 1972). The DTS is derived from the endoplasmic reticulum of megakaryocytes and is responsible for the sequestering of calcium that triggers contractile events (Ebbeling et al. 1992). In addition, it provides the location for enzymes involved in prostaglandin synthesis (Gerrard et al. 1976).

Platelets also contain many organelles including mitochondria, lysosomes, peroxisomes, dense bodies, and granules. Platelet mitochondria provide platelets with the energy required to carry out many of their complex processes such as

platelet secretion, shape change, and aggregation. Lysosomes of platelets contain hydrolases that are responsible for clot retraction. Peroxisomes contain catalase while platelet dense bodies contain serotonin (White 1968 and 1969), adenine nucleotides (Wojenski and Schick 1993), adrenaline and noradrenaline, histamine and dopamine (Holmsen and Weiss 1979). Furthermore, recent evidence suggests that platelet dense bodies may also contain spare receptors such as GP IIb/IIIa and GP Ib (Youssefian et al. 1997). Platelet alpha granules contain fibrinogen (Broekman et al. 1975, Karpatkin et al. 1984), von Willebrand Factor (Wencel-Drake et al. 1985), fibronectin (Zucker et al. 1979), extra platelet receptors (Gogstad et al. 1981), and mitogenic factors such as platelet-derived growth factor (Kaplan et al. 1979, Ross 1979).

Due to the large number of granules in platelets, a major function of the platelet is secretion that is often referred to as the release reaction. The secreted products further mediate and amplify the aggregation of platelets, a process that is responsible for maintaining hemostasis by preventing blood loss. Amongst these products are factors that mediate the three currently known pathways of platelet aggregation, including: ADP, TXA_2, and MMP-2 (Sawicki et al. 1997) (Figure 6).

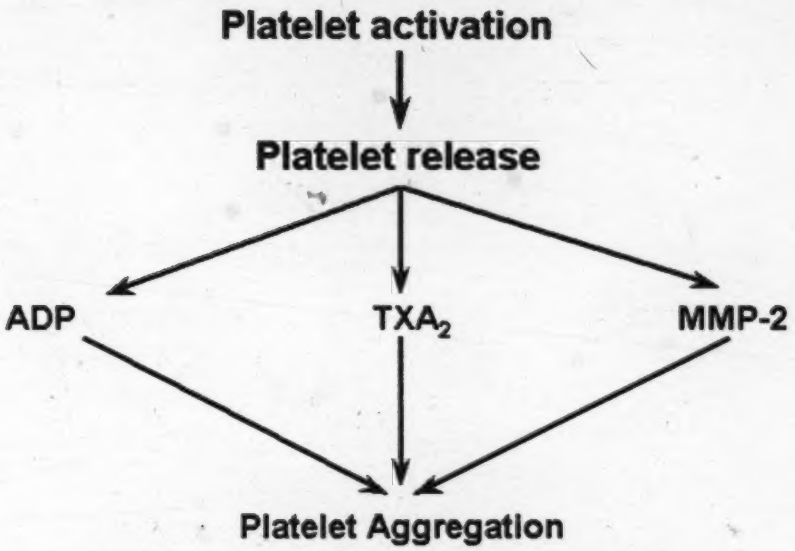

Figure 6. Major mediators of pathways of platelet aggregation.

These three factors along with other platelet ligands such as thrombin, collagens, and biogenic amines will bind to their respective receptors on the platelet membrane surface and activate the platelet (Figure 7). ADP, TXA_2, thrombin, and the biogenic amines bind to receptors that have 7 transmembrane domains and are G-protein coupled. This leads to activation of phospholipase C and signaling through phosphatidyl inositol pathways, which result in the release of intracellular stores of Ca^{2+} from the dense tubular system, and in the activation of protein kinase C (PKC) by diacyl glycerol (DAG) (Figure 7). PKC can than phosporylate a number of targets, most importantly GP IIb/IIIa, which upon phosphorylation becomes activated and may bind its ligand fibrinogen and lead to platelet aggregation. Increased levels of Ca^{2+} will lead to the release reaction, which causes the release of TXA_2, ADP, MMPs, TIMPs, extra receptors, and

biogenic amines. The release reaction takes place via the open canalicular system. Generation of TXA_2 takes place via the cyclooxygenase (COX) pathway. Platelet activation via collagen and cross-linking of Fc receptors leads to activation of PKC through tyrosine kinases. In addition, when Ca^{2+} levels spike, platelet nitric oxide (NO) is produced resulting in autocrine inhibition of platelet activation via a cGMP-dependent pathway. Moreover, paracrine stimulation of the adenylate cyclase platelet pathway by prostacyclin (PGI_2), released from the endothelium, leads to platelet inhibition (Figure 7).

In addition to mediating hemostasis, the platelet release reaction also plays an inflammatory role. Activated platelets release from α-granules a number of chemokines such as platelet factor 4 (PF-4) (Broekman et al. 1975), β-thromboglobulin (β-TG) (Moore et al. 1975), connective tissue activating peptide III (CTAP-III) (Castor et al. 1977), neutrophil-activating peptide (NAP-2) (Car et al. 1991), regulated on activation normal T cell expressed and secreted chemokine (RANTES) (Kameyoshi et al. 1992), and macrophage inflammatory protein 1 (MIP-1α) (Klinger et al. 1995). Platelet released chemokines serve as chemotactic and activation signals for monocytes and neutrophils. In addition, these substances aid in the binding of leukocytes to endothelium and in their extravasation. Furthermore, platelet α-granules contain cationic proteins (Nachman et al. 1970) that have numerous effects such as an anti-bacterial effect, pyretic effects, anticoagulant activity, increased vascular permeability, and increased PMN infiltration. Platelets also contain cytokines such as interleukin-1 (IL-1) (Hawrylowicz et al. 1989). In addition, thrombospondin, a platelet protein, is

a chemotactic signal for neutrophils (Mansfield et al. 1990). Platelet dense bodies also contain a number of substances that upon release are proinflammatory such as histamine and serotonin. Moreover, platelets may affect other inflammatory cells, as they can stimulate the neutrophil oxidative burst (Ruf and Patscheke 1995). Platelet activation by thrombin leads to expression and secretion of macrophage chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) from neutrophils (Neumann et al. 1997). Platelets may stimulate the production of leukotrienes in PMNs through the exchange of arachidonic acid metabolites (Fiore and Serhan 1990, Palmantier and Borgeat 1991). Platelets further express complement receptors (Devine 1992) and FcγRII receptors (Karas et al. 1982) on their surface. Cross-linking of platelet Fc-receptors leads to platelet activation and aggregation (Clark et al. 1982). In addition, the presence of IgE receptors on the platelet surface allows platelets to mediate an IgE-dependant killing mechanism (Joseph et al. 1985). Figure 7 summarizes a complex network of platelet-regulatory pathways.

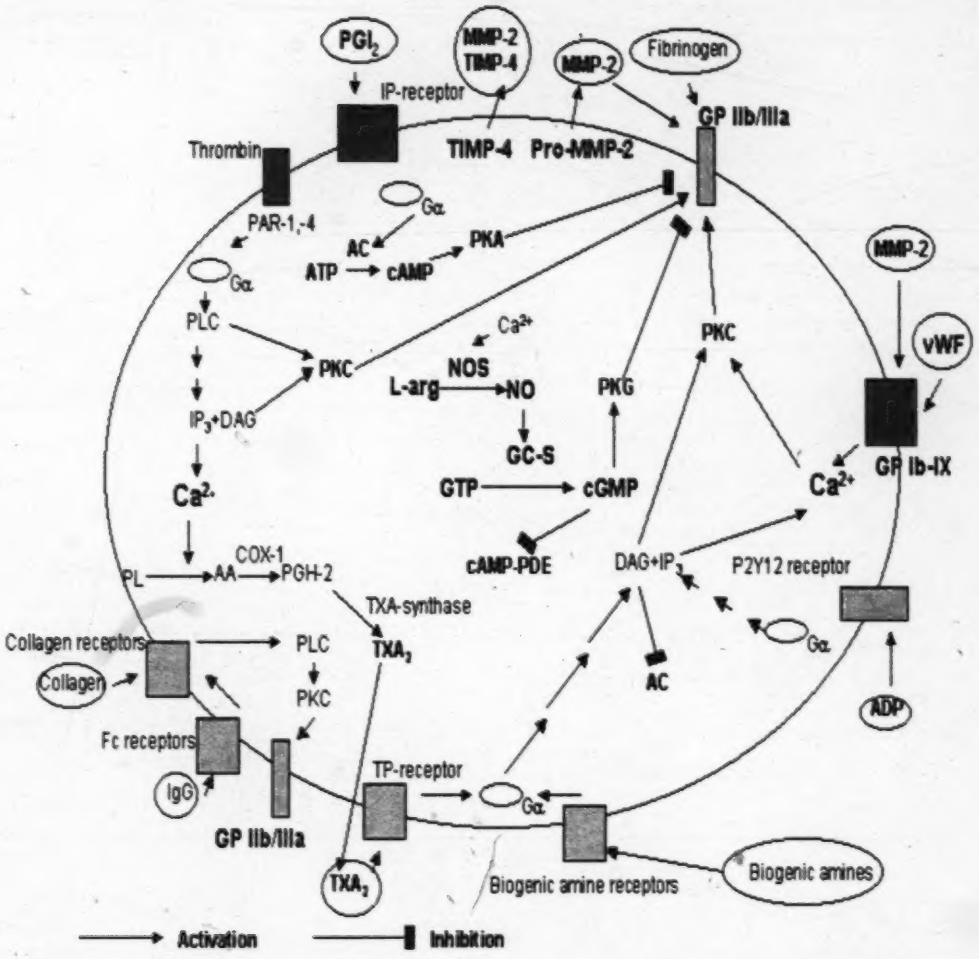

Figure 7. A summary of major platelet reactions and intracellular signaling pathways. The pathways with relevance to this thesis are highlighted in bold. Receptors are boxes and ligands are circled. Alpha subunit of a G-protein, Gα; arachidonic acid, AA; adenylate cyclase, AC; dense tubular system, DTS; Fc-receptor γ, FcRγ; glycoportein, GP; prostacyclin receptor, IP-receptor; thromboxane receptor, TP-receptor; mitochondria, MT; membrane phospholipids, PL; open canalicular system, OCS; protease activated receptor, PAR. Other abbreviations are defined in the list of abbreviations.

The Tumor Cell

Tumor is the Latin word for swelling. The Roman physician Celsus recognized swelling or tumor as one of the five cardinal signs of inflammation. Presently, the word "tumor" usually used to mean "cancer", which is a malignant neoplasm, or a new growth whose cells have undergone a change or transformation that allows them to escape normal homeostatic mechanisms that control cellular proliferation. Transformation from a normal cell to a tumor cell occurs due to mutations, that is, changes in the primary structure of a cell's DNA (this is also known as a lesion). These mutations can be as simple as a point mutation, that is, a change in a single base pair in the DNA chain. Transformation takes place when these mutations occur in proto-oncogenes, which then become oncogenes. Proto-oncogenes are genes that encode growth factor receptors, signal-transducing proteins, and nuclear regulatory proteins that are protein products of normal growth stimulatory genes. When these genes are damaged (mutated) so that their products are altered or over-expressed to promote tumor growth, they are called oncogenes. Transformation also occurs when mutations occur in genes that encode for tumor suppressor genes. Tumor suppressor genes encode proteins that are responsible for controlling transcription, cell cycle checkpoints and apoptosis. Furthermore, transformation is often also influenced by shifts in gene expression that are caused by epigenetic events. Epigenetic events that can lead to shifts in gene expression include DNA methylation, histone acetylation/deacetylation, and alterations in feedback loops for growth factors. Evidence indicates four to seven genetic events are required for the

development of epithelial cancers (Renan 1993), a process that may take many years.

Fundamentally, two types of neoplasms exist: benign and malignant. Benign neoplasms remain localized and do not spread to different parts of the body. Conversely, malignant neoplasms invade and destroy host tissue, and may spread throughout the body. According to Hanahan and Weinberg six essential cellular changes must occur for a cancer (malignant neoplasm) to develop. These six include: self sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000). These six changes will be discussed in terms of genetic changes in proto-oncogenes and tumor-suppressor genes in the process of carcinogenesis. While the genetic changes in cancer are many and complex, only a brief overview of some of the more common changes will be provided.

The first acquired capability of cancer, as discussed by Hanahan and Weinberg, is self-sufficiency in growth signals. Many mutations in mitogenic signals may lead to this ability of cancer cells. One such example is that of G-protein mutations in endocrine cancers. Pituitary tumors have been found to contain mutations in $G\alpha_s$ protein leading to constitutively active enzyme resulting in elevated cAMP levels that are mitogenic in endocrine cells (Lyons et al. 1990). Furthermore, many activating mutations in G-proteins have been shown in fibroblastic tumors (Dhanasekaran et al. 1998). Perhaps an even more important mutation in providing a tumor self-sufficiency in growth signals are the mutations

that occur in the monomeric G-protein Ras and lead to the stimulation of the mitogen activated protein (MAP) kinase mitogenic pathway (Yamamoto et al. 1999). Cell proliferation may be blocked by antigrowth signals in two ways: by being forced into the quiescent G_0 phase out of the cell cycle or by being induced to differentiate. Thus, a number of mutations occur in the cell cycle genes of tumor cells that allow them to escape these growth inhibitory signals, this constitutes the second acquired capability of cancer cells. These mutations occur in genes controlling the cell cycle, such as the genes in the retinoblastoma protein (pRb) pathway including cyclin-dependent kinases (CDKs) and cyclins (Weinberg 1995), and in the over expression of genes controlling cellular differentiation such as c-myc (Foley and Eisenman 1999). The decision of whether to go through the cell cycle and enter the synthesis phase (S-phase), or not, occurs at the restriction point (R-point) at the end of the G_1 phase of the cell cycle. This R-point is controlled by pRb, which when phosphorylated and inactivated by CDKs results in the decision to go through the cell cycle. Mutations that deregulate R-point transition are thought to occur in all human malignant tumors. Similarly, over expression of the oncogene c-myc can impair cellular differentiation and result in growth via the binding of transcription factors Myc to Max, which prevents differentiation (Hanahan and Weinberg 2000).

The third acquired ability of cancer is the ability of the tumor cells to evade apoptosis. Apoptosis is programmed cell death that is characterized by cell shrinkage, condensation of chromatin, membrane blebbing, cell fragmentation, and phagocytosis of the dead cell. Apoptosis is also thought to limit the

tumorigenic process. When a mutation occurs in a proto-oncogene that converts it into an oncogene, a cell tries to repair the mutation; however, if it is not successful at repairing the damage the cell will then undergo apoptosis. However, tumor cells have acquired the ability to evade apoptosis. This may occur due to mutations in genes that control apoptosis such as in the p53 tumor suppressor gene (Sigal and Rotter 2000). Mutations in p53 are thought to occur in over 50% of tumors (Kaelin 1999). Moreover, mutations in anti-apoptotic bcl-2 family of genes are all potentially oncogenic; more specifically bcl-2 expression is often elevated in a number of cancers such as non-Hodgkin's lymphoma and small-cell lung cancer (Reed 1999). Conversely, mutations that result in a decrease of activity of apoptosis-promoting genes may also occur. Indeed, pro-apoptotic members of the Bcl-2 family such as Bax, may be mutated in gastrointestinal cancer (Adams and Cory 1998). Finally, uncoupling of the proapoptotic signal needed to activate the cysteine proteases known as caspases that mediate apoptosis may also occur (Thornberry and Lazebnik 1998).

The fourth acquired capability of tumor cells is limitless replicative potential. It is thought that normal human cells have a limited amount of doubling potential, that is, they are able to divide up to 60-70 times before death of the cell (Hanahan and Weinberg 2000). The telomeres may be responsible for this "internal clock". Telomeres are sequences of DNA at the end of chromosomes that become shortened due to the inability of DNA polymerases to completely replicate the 3' ends of chromosomes. Roughly 50-100 base pairs of telomeric

DNA are lost with each cellular division. The shortened chromosomes then undergo end-to-end fusion with each other and ultimately this results in the death of the cell (Counter et al. 1992). However, cancer cells have acquired the ability to maintain their telomere length, thus they have acquired limitless replicative potential. This is accomplished due to the up regulation of the enzyme telomerase, which adds nucleotides to the ends of telomeric DNA.

The fifth acquired capability of cancer cells is sustained angiogenesis. As far back as 1971, it was realized that a tumor requires the formation of new blood vessels for growth (Folkman 1971). A tumor cannot grow to more than 1-2 millimeters in diameter without invoking the growth of these new blood vessels. This is due to the diffusion limit of oxygen and nutrients required by the tumor cells for growth. In a growing tumor, which is being vascularized, there is a an imbalance between promoters of angiogenesis such as vascular endothelial growth factor (VEGF), acidic/basic fibroblast growth factor (a/bFGF), $\alpha_v\beta_3$ integrins and matrix metalloproteinases (MMPs), and angiogenesis inhibitors such as interferons α/β, thrombospondin, platelet activating factor-4, 16Kda prolactin, angiostatin, and endostatin (Hanahan and Folkman 1996, Eliceiri and Cheresh 1999, Stetler-Stevenson 1999, and Sim et al. 2000). Novel therapeutic strategies based on inhibition of angiogenesis are now being developed and have been tested in clinical trials in the treatment of cancer. These include recombinant human angiostatin and endostatin, and gene transfer strategies of these two proteins, anti-VEGF receptor antibodies, and PEX, a MMP-2 hemopexin-domain derived fragment (Brooks et al. 1998, Hagedorn and Bikfalvi

2000). The contributing role of platelets to tumor angiogenesis requires careful study (Pinedo et al. 1998, Verheul and Pinedo 1998, Browder et al. 2000), as platelets are known to contain both angiogenesis promoters and inhibitors, and they are the largest circulating stores of VEGF and platelet factor-4 (Maloney et al. 1998, Gupta et al. 1995).

The sixth acquired capability of cancer cells is invasion and metastasis. These two processes are facilitated through altered expression of cellular adhesion molecules (CAMs), integrins, and extracellular proteases by cancer cells and other cells that the cancer cells may affect, such as stromal cells (Albelda 1993, Stetler-Stevenson et al. 1993, Stetler-Stevenson 1995, Himelstein et al. 1995, Crawford and Martisian 1995). A decrease in the adhesive properties between cells enables the cancer cell to be released from the primary tumor. Furthermore, degradation of the extracellular matrix by proteases will increase the ability of cancer cells to migrate and facilitate the metastatic process (Forget et al. 1999).

When the cancer spreads to another site of the body this is known as metastasis (Figure 8). In fact, it is metastasis that results in the death of an individual with cancer and not excessive cellular proliferation (Sporn 1996). Metastasis may occur in a number of ways. Cancerous cells may metastasize by surface implantation to body cavities or by the vascular route. Metastasis via the vasculature may occur in two different ways, either through lymphatic or hematogenous (blood-born) spread. Metastasis via the vasculature may be arbitrarily subdivided into five stages (Evans 1991). The first stage occurs when

Figure 8. A chest x-ray indicating the presence of a lung metastasis. An arrow indicates the metastatic lesion.

the tumor becomes malignant and invades local tissue. Next, the malignant tumor will intravasate into the vasculature. This stage is followed by vascular dissemination. Next, the malignant tumor cell(s) will arrest in the vasculature and extravasate, or leave the vasculature. Finally, the metastasizing cancer cell(s) will form a secondary growth or focus. If the metastasis through the vasculature occurs hematogenously the cancer cells will interact with a number of different cell types such as leucocytes and platelets. Due to the rheology of flowing blood, cancer cells will interact with platelets, as platelets are the first cells they will encounter, being closest to the endothelium (Jurasz et al. 2000). Platelet-cancer cell interactions are important in helping to mediate successful metastasis.

Mediators and Modulators of Platelet-Tumor Cell Reactions

Matrix Metalloproteinases

Proteases are enzymes that degrade other proteins. Proteases are subdivided into exopeptidases, which cleave one or a few amino acids off the N- or C-terminals of proteins and endopeptidases, which cleave proteins internally. Currently, endopeptidases are classified into four families based on their catalytic sites. The four families are the Serine proteases such as thrombin, Aspartic proteases such as pepsin, Cysteine proteases such as papain, and metalloproteases such as matrix metalloproteinases-2 (Barrett et al. 1998). A sub-family of the metalloproteases is the matrix metalloproteinases (MMPs), also known as matrixins. At the time that this thesis is being written, at least 20 human matrix metalloproteinases have been described. They are classified on the basis of their protein domain structures and substrate preference (Table 1). The structure of a prototypical MMP can be divided into four domains. The first is the propeptide domain. The propeptide domain contains a highly conserved sequence of eight amino acids that fold over to cover the catalytic site and maintain the protease in its inactive conformation. Thus, MMPs are secreted as zymogens requiring processing of the MMP molecule for enzymatic activity. The second domain is the catalytic domain. The catalytic domain contains a zinc-binding site, which is essential for catalytic activity. The Zn^{2+} ion is linked to two cysteine residues that stabilize the protease and keep it in its inactive form. Enzymatic cleavage of these two cysteine residues results in the activation of the MMP.

Collagenases:	Substrates
MMP-1 (interstitial collagenase)	collagens I,II,III, VII, VIII, X, gelatin, aggrecan, tenascin
MMP-8 (neutrophil collagenase)	collagens I,II,III, VII, X, aggrecan
MMP-12 (metalloelastase)	proteoglycans, laminin-1, elastin, fibronectin, collagen IV, entactin
MMP-13 (collagenase-3)	collagens I,II,III, VII, X, aggrecan, gelatin
Gelatinases:	
MMP-2 (gelatinase A, collagenase IV)	gelatin, fibronectin, collagens I,II,III, IV, V, VII, X, XI, aggrecan, laminin
MMP-9 (gelatinase B)	gelatin, fibronectin, collagens II, IV, V, VII, X, XI, aggrecan, vitronectin
Membrane-Type:	
MMP-14 (MT1-MMP)	MMP-2, MMP-13, laminin, gelatin, fibronectin, collagens II, IV, V, VII
MMP-15 (MT2-MMP)	MMP-2, laminin, gelatin, fibronectin, collagens I, III, aggregan, tenascin
MMP-16 (MT3-MMP)	MMP-2, gelatin
MMP-17 (MT4-MMP)	MMP-2, gelatin
MMP-24 (MT5-MMP)	
MMP-25 (MT6-MMP)	
Stromelysins:	
MMP-3 (stromelysin-1)	proteoglycans, laminin, gelatin, fibronectin, collagens, I, III, aggrecan
MMP-10 (stromelysin-2)	proteoglycans, laminin, fibronectin, aggrecan, elastin, collagens
MMP-11 (stromelysin-3)	casein
Other:	
MMP-7 (matrilysin)	proteoglycans, laminin, fibronectin, gelatins, collagen IV, elastin
MMP-19	aggrgcan
MMP-20	amelognin
MMP-26 (matrilysin-2)	

Table 1. Human matrix metalloproteinases

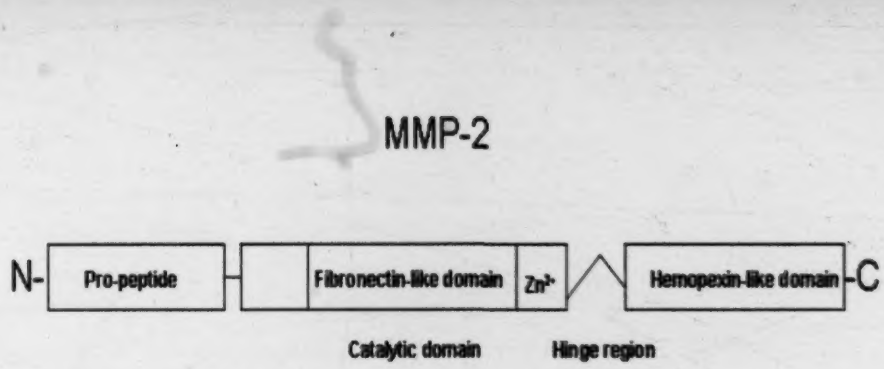

Figure 9. Schematic representation of MMP-2; a member of the gelatinase subfamily of matrix metalloproteinases.

A third important domain of MMPs is the hinge region; it has been shown to confer substrate specificity (Hirose et al. 1993) in addition to separating the catalytic domain from the hemopexin-like domain. The gelatinase sub-class of MMPs also contains a fibronectin-like domain in addition to a hemopexin-like domain (Figure 9). This fibronectin-like domain is involved in the recognition of elastin (Shipley et al. 1996). In addition, members of the membrane-type class of MMPs also contain a short C-terminal transmembrane domain (Sato et al. 1997). Some of the biochemical characteristics shared by all MMPs include the requirement of Ca^{2+} and Zn^{2+} for enzymatic activity and a physiological pH for optimal activity.

MMPs play major roles in both physiological and pathological processes. Physiologically, MMPs are involved in processes that require remodeling of tissues and extracellular matrix such as reproduction and embryogenesis (Salamonsen et al. 2000), bone and tooth enamel development (Bord et al. 1997), wound healing, angiogenesis (Stetler-Stevenson 1999), and platelet

aggregation (Sawicki et al. 1997). Pathologically, MMPs have been implicated in many diseases such as osteoarthritis (Dean et al. 1989), rheumatoid arthritis (McCachren et al. 1991), multiple sclerosis (Beaver and Rosenberg 1999), Alzheimer disease (Yong et al. 1998), ischemia-reperfusion injury of the heart (Cheung et al. 2000), and cancer (Liotta et al. 1980).

An enzymatic cascade activates most MMPs. Urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator cleave and convert plasminogen into plasmin. Then plasmin, or other serine proteases such as elastase and trypsin, activate the pro-MMP by cleaving at one of the cysteine residues that stablize the catalytic site of the MMP. A molecule of H_2O stabilizes this reaction. The second step in the activation process involves the autocatalytic cleavage of the MMP for full activity. This second step may be inhibited by endogenous inhibitors of MMPs, the tissue inhibitors of matrix metalloproteinases (TIMPs). However, activation of MMP-2 occurs via a different mechanism in which a ternary complex containing pro-MMP-2, TIMP-2, and two molecules of membrane-type MMPs forms on the outer cellular membrane surface. The pro-domain of pro-MMP-2 is cleaved by one of the molecules of MT-MMP-1 leading to the activation of MMP-2; furthermore, this reaction is stabilized by TIMP-2 and the second MT-MMP (Forget et al. 1999).

MMPs play an important role in cancer and in platelet aggregation. Matrix metalloproteinases, when released by tumor cells, degrade the basement membrane, thus facilitating metastasis (Liotta et al. 1980, Liotta et al. 1986, Ray et al. 1994). In platelets, the release of MMP-2 has been shown to mediate a

novel pathway of platelet aggregation (Sawicki et al. 1997). During aggregation MMP-2 is translocated to the platelet surface membrane (Sawicki et al. 1998). Aggregation-stimulating effects of MMP-2 may depend on its interactions with GP Ib and GP IIb/IIIa (Martinez et al. 2001, Radomski et al. 2001). Furthermore, MMP-9 has been shown to counteract the pro-aggregating effects of MMP-2 by inhibiting platelet aggregation (Fernandez-Patron et al. 1999).

Inhibitors of Matrix Metalloproteinases

A number of endogenous and synthetic inhibitors control the enzymatic activity of MMPs. The endogenous inhibitors of MMPs include the tissue inhibitors of matrix metalloproteinases (TIMPs) and β-macroglobulin.

The TIMPs are a small family of MMP inhibitors that regulate the activity of MMPs. Currently there are four known members of the TIMP family (TIMP-1 to TIMP-4) and they share a high level of sequence homology. TIMPs are small proteins ranging from 20-30 kDa in size and consist of two domains. The N-terminal domain is about 125 amino acids in length and the C-terminal domain consists of 65 amino acids (Williamson et al. 1990). Twelve cysteine residues form 6 disulfide bonds that stablize the TIMP-MMP complex. The N-terminal domain binds the Zn^{2+} containing catalytic site of MMPs and this domain alone can bind and inhibit MMPs (Murphy et al. 1991). Studies with MMP-3 and TIMP-1 showed that the α-amino and carbonyl groups of the N-terminal cysteine of TIMP-1 interact with the Zn^{2+} containing catalytic site to inhibit MMP-3 (Gomis-Ruth et al. 1997). The C-terminal domain of some TIMPs also binds to and is

involved in the recognition of some MMPs, especially MMP-2 and MMP-9 (Fridman et al. 1992). Furthermore, the binding of TIMPs to MMPs occurs with high affinity. Biggs et al. have shown the apparent K_i of TIMP-4 for MMP-2 and MT1-MMP for example to be in the pM range (Biggs et al. 2001).

While most TIMPs bind most MMPs, differences do exist among TIMPs both in physical properties and in specificity. TIMP-1 and –2 are soluble, while TIMP-3 binds to the extracellular matrix (ECM) tightly and is insoluble. Furthermore, both TIMP-2 and TIMP-3 are good inhibitors of MT-MMPs, but TIMP-1 is not. Moreover, both TIMP-2 and TIMP-4 effectively bind to MMP-2 via their C-terminal domains. Thus, due to their differences in specificity TIMPs have distinct physiological functions. For example, TIMP-2 is involved in the activation of pro-MMP-2. Moreover, TIMPs also have distinct non-MMP-inhibitory functions. It has been shown that TIMP-2 stimulates cell growth of kidney mesenchymal cells (Barasch et al. 1999); while TIMP-3 induces apoptosis in SK-Mel-5 and A2058 melanoma, and HT-1080 fibrosarcoma cell lines (Ahonen et al. 1998, Baker et al. 1999).

Traditionally, the TIMPs have been known to function in maintaining a balance in the turnover of the extracellular matrix during tissue remodeling. Thus, the expression of TIMPs during remodeling is tightly controlled. Disruption in the control of TIMP expression leads to an imbalance in the turnover of the ECM and may result in disease (Jackson et al. 1998 and Mohanam et al. 1995). For example, this may result in tumor cell invasion and metastasis if the balance tips

towards activated proteases due to a decrease in endogenous inhibitors such as the TIMPs (Stetler-Stevenson et al. 1993)

Other endogenous inhibitors of MMPs include α_2-macroglobulin (Welgus and Stricklin 1983); however, this large protein is a non-specific protease inhibitor.

A number of synthetic agents inhibit the actions of MMPs. These include such compounds as o-phenanthroline, doxycycline, Batimastat®, and Marimastat®. Ortho-phenanthroline is a synthetic inhibitor limited in use to the laboratory. The antibiotic doxycycline has also been shown to inhibit MMP activity (Greenwald et al. 1992). Interestingly, Golub and colleagues developed chemically-modified tetracyclines that are devoid of anti-bacterial properties, yet they are potent inhibitors of MMPs (Golub et al. 1999). The first compounds developed with the specific goal to inhibit MMPs were Batimastat (Davies et al. 1993) and Marimastat (Wojtowicz-Praga et al. 1998). These two inhibitors are low-molecular weight compounds with a hydroxymate structure that mimics that of the collagen-binding site (Wojtowicz-Praga et al. 1997). The mechanism of action of all currently known MMP inhibitors is thought to be due to the chelation of the Zn ion in the active site of the MMP. Unfortunately, clinical trials of MMP inhibitors for the treatment of cancer to date have not shown MMP inhibitors to be clinically efficacious. For further information on the reasons for the failure of these compounds as anti-cancer drugs please see articles by Greenwald 1999 and Zucker et al. 2000.

Nitric Oxide

Nitric oxide (NO) is a simple, yet one of the most important signaling and effector molecules in the human body. This diatomic, gaseous molecule plays important roles mediating diverse biological processes such as vasorelaxation, immunity, neurotransmission, and inhibition of platelet aggregation. Identified for the first time by Furchgott and Zawadzki, in 1980, for its endothelium dependant vasorelaxing effects, it was initially named endothelium-derived relaxing factor (EDRF) because its chemical nature was unknown. EDRF was early described as a potent stimulator of the soluble guanylate cyclase (GC-S) in a variety of tissues (Arnold et al. 1977); however, it was not until some ten years later when it was found that the release of NO could account for the vasorelaxant properties of EDRF (Palmer et al. 1987, Ignarro et al. 1987).

Nitric oxide is synthesized by the enzyme nitric oxide synthase (NOS) (Palmer and Moncada 1989). Currently, three isoforms of this enzyme have been cloned, including endothelial NOS (eNOS), neuronal NOS (nNOS), and cytokine-inducible NOS (iNOS) (Griffith and Stuehr 1995) (Figure 10). Both eNOS and nNOS are Ca^{2+}-dependant due to Ca^{2+}/calmodulin (CaM) binding to these isoforms, therefore they produce bursts of NO when cellular Ca^{2+} levels spike. Removal of Ca^{2+} from NOS using chelating agents, such as EDTA, leads to the dissociation of CaM and inhibition of nNOS and eNOS activities. In contrast to eNOS and nNOS, iNOS binds calmodulin tightly so that it does not dissociate. This lack of Ca^{2+} control over iNOS results in constant production of large amounts of NO when iNOS is induced, during various immunologic and

inflammatory reactions. In addition, all three NOS isoforms require a number of cofactors for enzymatic activity including: NADPH, FAD, FMN, and tetrahydrobiopterin (THB_4) (Palmer and Moncada 1989, Griffith and Stuehr 1995).

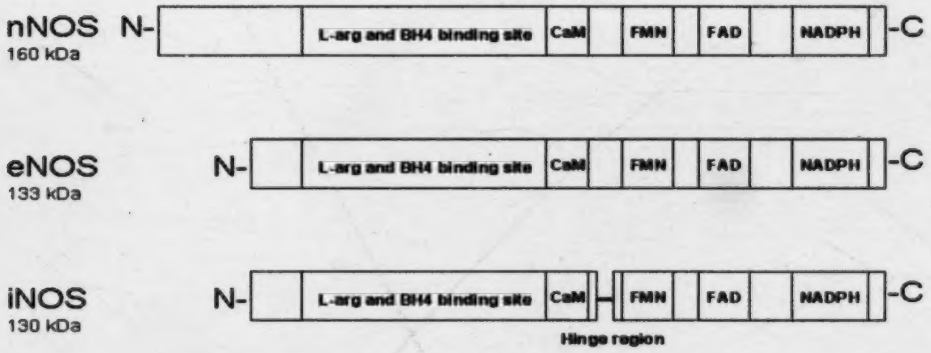

Figure 10. The three isoforms of nitric oxide synthase. nNOS = neuronal, eNOS = endothelial, and iNOS = inducible.

The oxidation-reduction reactions carried out by NOS result in the conversion of L-arginine (L-arg) and two molecules of O_2 into NO, L-citrulline, and two molecules of H_2O (Figure 11). Once formed, NO may then act in an autocrine or, due to its high lipid solubility, paracrine fashion. NO mediates its signaling via binding to GC-S, which results in the activation of this enzyme and the production of the second-messenger cGMP (Figure 11). Cyclic GMP may then act on cGMP-gated ion channels, cGMP-inhibited/stimulated cAMP-phosphodiesterases to augment cAMP responses, or cGMP-dependent protein kinase (PKG). In

addition, NO may act in a non-cGMP manner due to its high reactivity. It may nitrosylate thiols in proteins, or it may react with radical species such as superoxide (O_2^-) to form peroxynitrite ($ONOO^-$) (Moro et al. 1994).

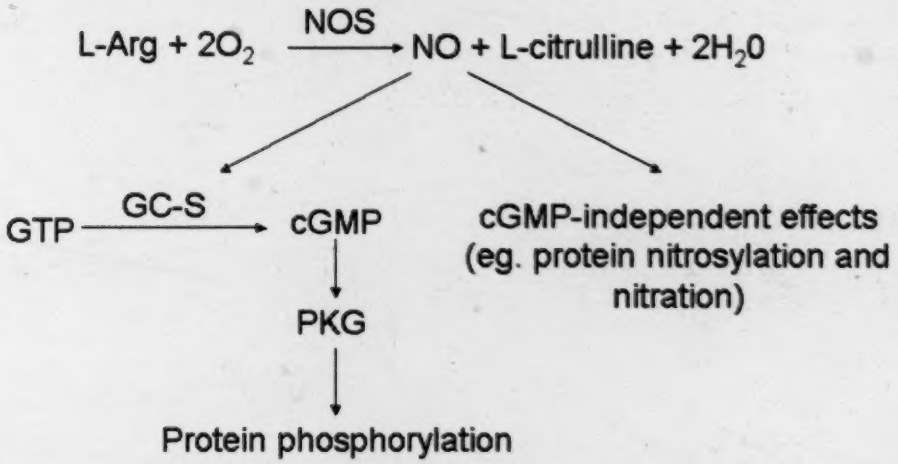

Figure 11. Formation of NO from L-arg by NOS. NO may then act in a cGMP-dependent or -independent manner.

A number of inhibitors of the NOS enzymes have been described. These include both arginine analogues and non-arginine analogue inhibitors. Some of the more common arginine analogues include: N-monomethyl-L-arginine (L-NMMA), N-nitro-L-arginine methyl ester (L-NAME), N-nitro-L-arginine (L-NA), and N-amino-L-arginine (L-NAA). These compounds act as competitive inhibitors of NOS, as they decrease enzyme activity acting at the arginine-binding site of the NOS enzymes. These inhibitory effects are reversed by an excess of L-arg. Non-arginine analogue inhibitors include compounds such as aminoguanidine that was reported to be a relatively selective-inhibitor of iNOS (Misko et al. 1993). In

addition to NOS inhibitors, NO action can be inhibited by NO scavenging agents such as hemoglobin (Sharma et al. 1987) and some oxido-reducing dyes such as methylene blue (Radomski et al. 1988).

Nitric oxide through a cGMP-dependent mechanism has the capacity to modulate the cardiovascular system. When NO diffuses out from endothelial cells lining a blood vessel it may reach vascular smooth muscle cells resulting in dilatation of the blood vessel (Figure 12). The diffusion of NO into the vascular lumen regulates platelet function. Indeed, NO may inhibit both platelet adhesion to the endothelium (Radomski et al. 1987a, Radomski et al. 1987b), and subsequent platelet aggregation (Radomski et al. 1987c, Radomski et al. 1987d). In addition, platelets have the capacity to generate NO that regulates aggregation (Radomski et al. 1990a and 1990b). Malinski et al. using a washed platelet preparation have reported that human platelets produce up to $10.5\pm2.4 \times 10^{-18}$ mole of NO per platelet during aggregation with collagen (Mainski et al. 1993). Moreover, inhibition of platelet aggregation by nitric oxide also occurs through a cGMP-mediated mechanism (Moro et al. 1996). In addition, pharmacological NO donors such as S-nitrosoglutathione (GSNO) have been shown to inhibit platelet aggregation at concentrations that do not exert a significant effect on vascular relaxation (Radomski et al. 1992, de Belder et al. 1994). Other NO donors include S-nitroso-acetyl-penicillamine (SNAP), the organic nitrates such as glyceryl trinitrate, inorganic nitroso compounds such as sodium nitroprusside, and syndomines such as molsidomine (SIN-1) (Moncada and Higgs 1995).

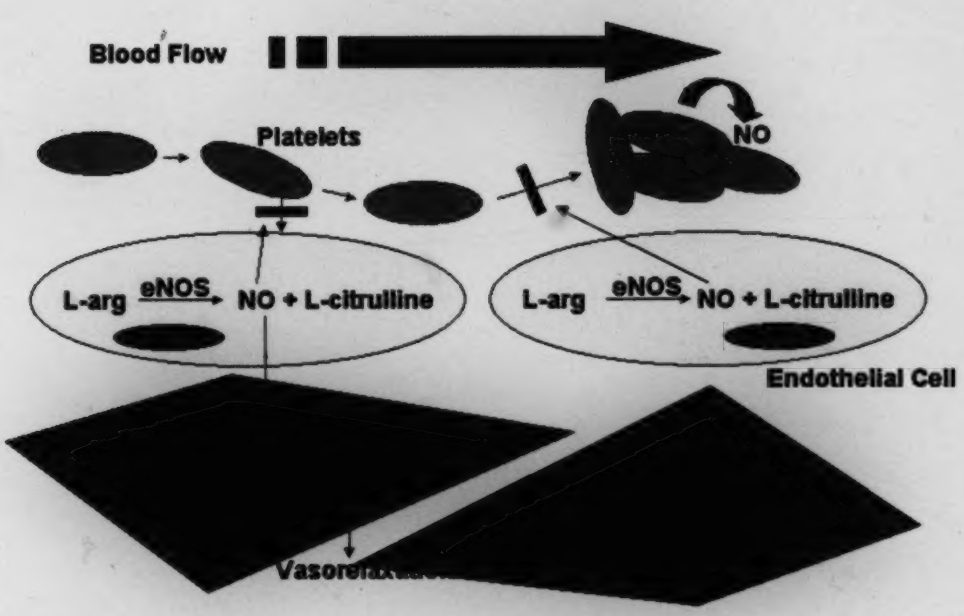

Figure 12. Nitric oxide formation and effects on the vascular wall and platelets. Arrows denote stimulation and bars inhibition.

Furthermore, NO has the ability to modulate tumor cell-induced platelet aggregation. It has been shown that human colon carcinoma cells isolated from metastases exhibited lower NOS activity than cells isolated from the primary tumor, and the metastatic cells were more potent inducers of platelet aggregation (Radomski et al. 1991). In addition to TCIPA, NO has been identified to mediate numerous, and often opposing, roles in processes such as tumor progression (Morcos et al. 1999), neovascularization (Jenkins et al. 1995), immunity (Stuehr et al. 1989), and apoptosis (Binder et al. 1999).

Prostaglandins and Thromboxane

Prostaglandins, first identified in the 1930s, have many physiological effects, as they are involved in inflammatory reactions, the production of pain and fever, the regulation of blood pressure, the control of reproductive functions, and the induction and inhibition of platelet aggregation. The enzyme responsible for prostaglandin production is cyclooxygenase (COX). Currently, it is known that there are two isotypes of cyclooxygenase: COX-1 and COX-2. COX-1 has been traditionally known as the constitutively expressed form of the enzyme and it is found in high amounts in cells such as endothelial cells and platelets. It is a membrane bound homodimer 72 KDa in size, and it requires heme for enzymatic activity (Hemler et al. 1976). COX-2, on the other hand, is the inducible form of the enzyme that is responsible for many of the inflammatory reactions (Mitchell and Warner 1999). However, recent evidence suggests that two isoforms of COX have overlapping functions in that they both have roles in physiological processes and in inflammation (Wallace 1999). COX has both cyclooxygenase and peroxidase activity, whereby it converts the lipid metabolite arachidonic acid to prostaglandin H_2 (PGH_2) (Smith 1992) (Figure 13). After the conversion of arachidonic acid to PGH_2 by cyclooxygenase a number of other synthases convert PGH_2 to prostaglandins such as PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), and thromboxane A_2 (TXA_2) (Figure 13).

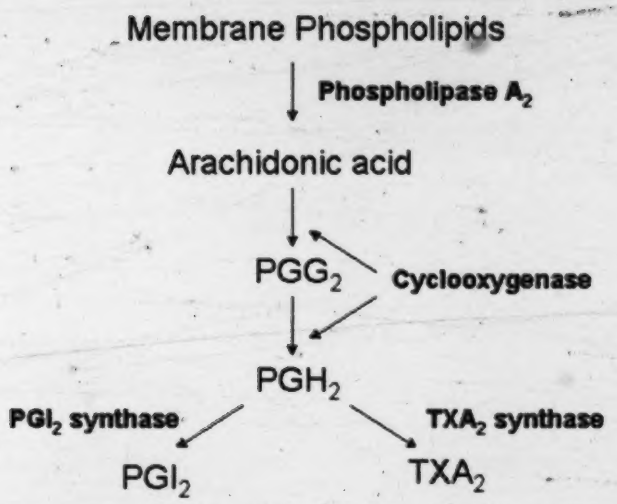

Figure 13. The reactions leading to the synthesis of prostaglandins and thromboxane.

Although prostaglandins are common products of the COX pathway and are formed from a common intermediate, a given cell type usually has one prostaglandin formed as the major product (Smith et al. 1991). Therefore the production of prostaglandins is said to be "cell-specific". In platelets, a major arachidonic acid product is TXA_2 (Hamberg et al. 1975), as platelets contain the enzyme thromboxane synthase; while in endothelial cells this major product is PGI_2 (Moncada et al. 1976). It is these two arachidonic acid metabolites that are proposed to be the most important lipid derivatives in regulating hemostasis, as they have opposing hemostatic effects (Moncada and Vane 1979a). While, TXA_2 promotes thrombosis by aggregating platelets and constricting blood vessels, PGI_2 is anti-thrombotic, as it inhibits platelet aggregation and dilates blood vessels. These two "unstable" prostaglandins have very potent and fast acting

actions, as they undergo rapid hydrolysis and have very short half-lives (0.5-5 minutes) (Hamberg et al. 1975, Moncada and Vane 1979b).

Thromboxane A_2 mediates platelet aggregation by stimulating platelet thromboxane receptors known as TP-receptors leading to activation of platelet inositol phosphate pathways and an increase in intracellular Ca^{2+} (Reilly and Fitzgerald 1993). An increase in intracellular Ca^{2+} leads to platelet activation, shape change, release of dense- and α-granules, exposure of fibrinogen receptors, and platelet aggregation (Armstrong 1996). Aspirin reduces platelet aggregation by inhibiting platelet cyclooxygenase and thereby inhibiting TXA_2 production. Although platelets do retain some mRNA from megakaryocytes and have some translating capacity, they are anucleate and are unable to synthesize new mRNA; consequently, they lack the capacity to synthesize new proteins such as COX (Roth et al. 1989). Therefore, the acetylation of platelet COX strongly inhibits its enzymatic activity and consequently TXA_2 production, an effect lasting for the entire life span of the platelet (Burch et al. 1978). Since the major arachidonic acid product formed by platelets is TXA_2, and it mediates one of the pathways of platelet aggregation, its inhibition of production by aspirin is responsible for aspirin's anti-thrombotic effects. At the same time, aspirin also inhibits COX in endothelial cells and thereby inhibits PGI_2 production. However, endothelial cells are nucleated and have the capacity to generate new enzyme. Therefore, endothelial cells have the ability to replenish their COX and are able to recover from aspirin treatment in a few days.

PGI_2 mediates its platelet inhibitory effects by binding to platelet IP-receptors (Armstrong 1996). The IP-receptor has seven transmembrane domains and it is a G-protein linked receptor (Figure 14). The G-protein then activates adenylate cyclase (AC) that converts ATP into cAMP. Inhibition of platelet aggregation by PGI_2 is mediated by cAMP-dependent mechanisms such as reduction of intraplatelet Ca^{2+} levels and down-regulation of platelet receptor function (Moncada and Vane 1979b, Graber and Hawiger 1982). In addition, to agonist induced platelet aggregation, PGI_2 has also been shown to be a potent inhibitor of TCIPA (Menter et al. 1984) and a potent antimetastatic agent (Honn et al. 1981).

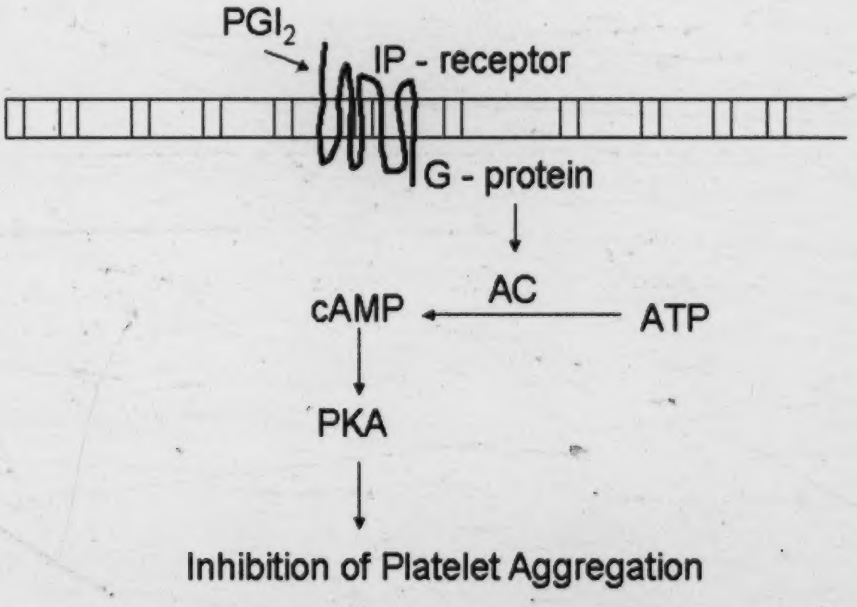

Figure 14. Role of cAMP in the platelet aggregation-inhibitory effects of PGI_2.

Platelet Glycoprotein IIb/IIIa and Ib Receptors

GP IIb/IIIa ($\alpha_{II}\beta_3$) is a platelet specific surface transmembrane receptor that belongs to the integrin superfamily (Figure 15).

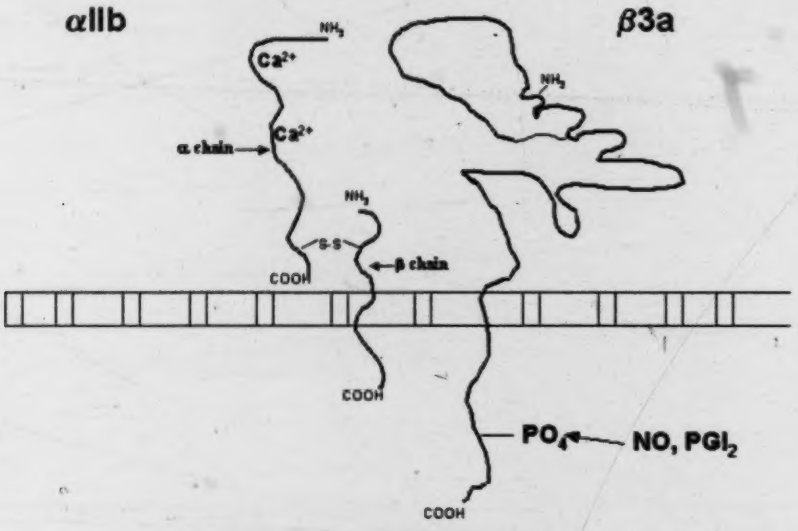

Figure 15. Schematic representation of platelet GP IIb/IIIa

It is the most abundant platelet receptor with roughly 80,000 copies on the surface of each platelet. In addition, platelets contain a pool of GP IIb/IIIa stored in platelet α granules (Wagner et al. 1996) that is released onto the platelet surface upon platelet activation. GP IIb/IIIa is composed of two subunits, a two-chain (a light chain and a heavy chain) α subunit and a single chain β subunit. The two subunits are noncovalently bound to each other and require Ca^{2+} to maintain their heterodimeric structure. The α subunit is 136-kd in size, while the β subunit is 92 kd (Leifkovits et al. 1995). The majority of the protein is extracellular and this includes the N-termini of both subunits.

The transmembrane domains of each subunit pass through the membrane once and each is linked to a short C-terminal cytoplasmic tail (Shattil et al. 1998). GP IIb/IIIa has a number of physiological ligands such as fibronectin and vitronectin; however, GP IIb/IIIa's primary ligands are fibrinogen and von Willebrand Factor. All these ligands contain the classical integrin recognition sequence Arg-Gly-Asp (RGD).

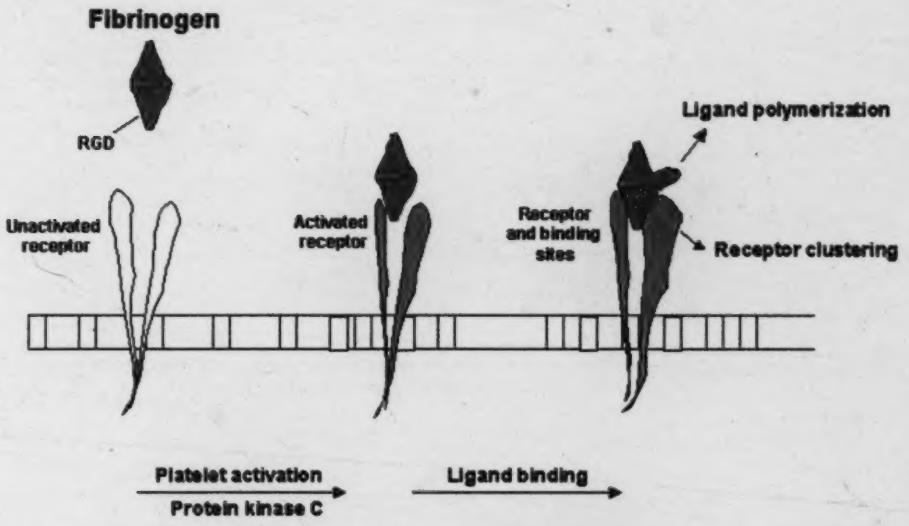

Figure 16. Activation of platelet receptor GP IIb/IIIa.

Furthermore, GPIIb/IIIa undergoes activation and a conformational change that allows binding to its primary ligands fibrinogen and vWF (Ruggeri et al., 1999) to mediate platelet-platelet and platelet-vessel wall interactions (Figure 16).

In 1983, Coller et al. developed the first GP IIb/IIIa receptor antagonist (Coller et al. 1983). They generated a murine monoclonal antibody named m7E3 that prevented fibrinogen from binding to its receptor GP IIb/IIIa thereby inhibiting

platelet aggregation. The first GP IIb/IIIa antagonist used in clinical trials and approved for treatment of acute coronary syndromes was abciximab (c7E3) (ReoPro, Centocor and Eli Lilly and Co.) (EPIC investigators 1994). This drug is a chimeric antibody Fab fragment that contains the original variable region of the m7E3 murine antibody. The murine variable region is joined to a constant region of a human IgG Fab (Madan et al. 1998). Since the initial development of abciximab, many other GP IIb/IIIa receptor antagonists have gone into clinical use for the treatment of acute coronary syndromes including orally active compounds. Many of these drugs are based on the disintegrins, a class of proteins found in the venoms of viper snakes that contain the RGD-sequence and interfere with the binding of RGD-containing proteins to integrins (Musial et al. 1990). For more information on GP IIb/IIIa antagonists please see reviews by Kleiman et al. 1998 and Madan et al. 1998. Furthermore, blockade of GP IIb/IIIa has been also shown to inhibit experimental TCIPA (Jurasz et al. 2001a). In addition, abciximab (c7E3) has been shown to possess inhibitory action against the growth of human mammary adenocarcinoma tumors xenotransplanted into nude mice (Trikha and Nakada 2000).

GP Ib belongs to the leucine-rich superfamily of receptors (Figure 17). It complexes with glycoproteins IX and V and forms an active receptor. The major ligand of GP Ib is vWF and its binding to the receptor mediates platelet adhesion and blood arrest at the damaged fragment of the vessel wall. However, shear forces generated under conditions of flowing blood may also result in vWF-mediated platelet aggregation (Ruggeri 1999).

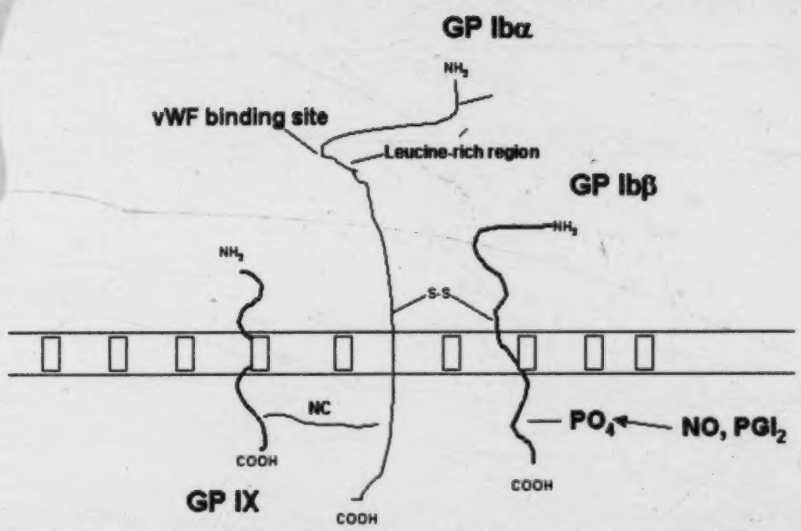

Figure 17. Schematic representation of platelet receptor glycoprotein Ib and IX. NC, non-covalent binding.

Agonist-induced platelet surface receptor expression can be modulated by a number of anti-platelet agents. Nitric oxide has been shown to affect GP IIb/IIIa receptor expression during platelet activation (Salas et al., 1994). Moreover, PGI_2 prevents the mobilization of platelet GP IIb/IIIa in thrombin-activated platelets (Graber and Hawiger, 1982). In addition, it has been shown that GP Ib/IX/V can be modified by the phosphorylation of Ser166 via cyclic AMP-dependent kinase (Wyler et al., 1986 and Wardell et al., 1989) that is activated by PGI_2.

von Willebrand Factor

von Willebrand Factor is a major adhesion protein that mediates platelet attachment to the vascular wall and this protein can interact both with GP Ib and GP IIb/IIIa receptors (Figure 18). It has the RGD-recognition sequence that is required for interaction with GP IIb/IIIa. VWF is synthesized in megakaryocytes and endothelial cells. It is a large multimeric protein found free in plasma, stored in platelet α-granules and Weibel-Palade bodies of endothelial cells, and immobilized in the subendothelium (Coller 1992). In its monomeric form, vWF has a molecular weight of 220 KDa. Monomeric forms of vWF then dimerize, and then these dimers undergo multimerization. Large vWF multimers may range from 880 to 20,000 KDa. Free vWF can cause platelets to agglutinate without aggregation. Platelet agglutination is a non-metabolic process that results in platelet clumping, while platelet aggregation is an active process that requires the platelet to spend energy. In addition, vWF is thought to mediate platelet aggregation during conditions of high shear force. A number of disorders have revealed the role of vWF *in vivo*. Von Willebrand disease and Bernard Soulier-syndrome are two hemorrhagic disorders that have shed light on the important role of vWF in hemostasis. In von Willebrand disease individuals have a deficiency of this factor and have a serious bleeding disorder that can be corrected with the administration of vWF (Ruggeri 1994). In Bernard-Soulier syndrome patients do not express GP Ib, the major platelet receptor for vWF (Lopez et al. 1998). These patients also have a severe hemorrhagic disorder. Moreover, the inability to cleave multimeric forms of vWF results in thrombotic

thrombocytopenic purpura (TTP) (van der Plas et al. 1999). The large multimeric forms of vWF result in both pathological thrombosis and also in bleeding due to the consumption of platelets. Moreover, this inability to cleave multimeric vWF has been found to be as a result of a deficiency of a metalloprotease (Furlan et al. 1998).

Recently, Stewart et al. (1997) reported that vWF, when immobilized on polystyrene beads, acts as a novel solid-phase agonist (sVWF) in human platelet-rich plasma (Figure 18). The development of this preparation has allowed me to study the interactions between vWF, platelets, and cancer cells *in vitro*.

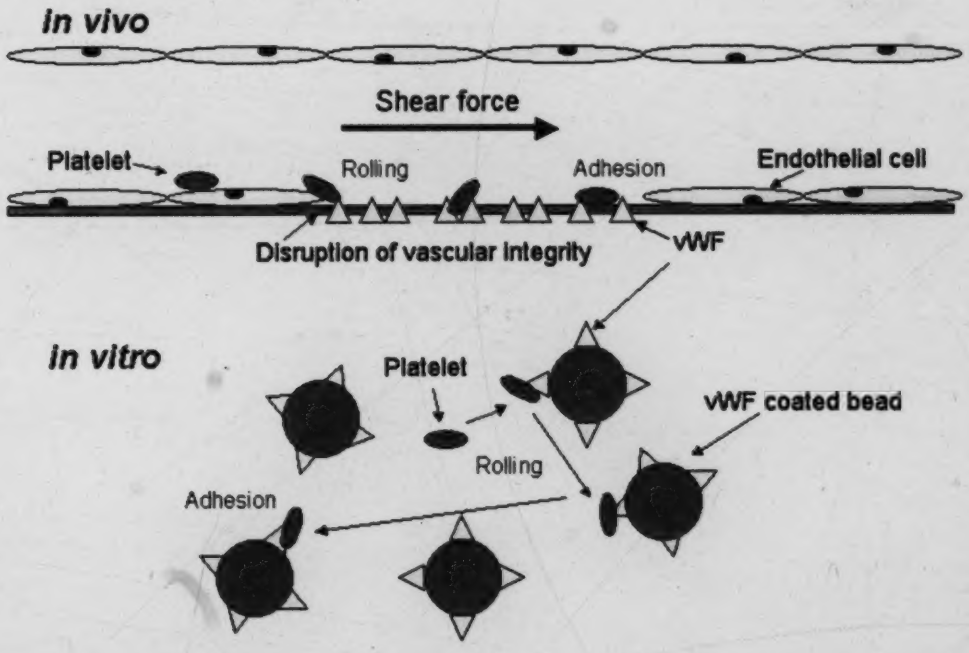

Figure 18. Schematic representations of the role of vWF *in vivo* and *in vitro*. *In vivo*, vWF mediates the interactions between platelets and the vascular wall. Note that vWF immobilized on beads mimics the *in vivo* reactions stimulated by the ligand.

Thesis Objectives and Aims

The overall objective of this dissertation was to study mechanisms of tumor-cell-induced platelet aggregation *in vitro*.

The aims of this research were as follows:

1. To investigate the involvement and regulation of matrix metalloproteinase-2 in tumor cell-induced platelet aggregation.
2. To investigate the regulatory effects of nitric oxide and prostacyclin in tumor cell-induced platelet aggregation.
3. To study the effects of solid-phase von Willebrand factor on tumor cell-induced platelet aggregation.
4. To measure the effects of tissue inhibitor of matrix metalloproteinase-4 on tumor cell-induced platelet aggregation.

Materials and Methods

Blood Platelets. This study was approved by the Ethics Committee of the Faculty of Medicine and Dentistry at the University of Alberta. Blood was collected from healthy human volunteers who had not taken any drugs for 14 days prior to the study. Washed platelet suspensions ($2.5x10^{11}$/L) were prepared as previously described (Radomski et al. 1983).

Tumor Cell Culture. Two human tumor cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). Cell lines were cultured as monolayers in 250 ml culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 in air. The HT-1080 and A549 cell lines were cultured in 90% Dulbecco's Modified Eagle's Medium (DMEM) with non-essential amino acids, gentamicin (0.05 mg/ml), penicillin (0.06 mg/ml), streptomycin (0.01 mg/ml), and with 10% fetal bovine serum (FBS). The cells were supplied with fresh medium and subcultured 3 times each week. Cells were detached from the flasks using EDTA (7mM) in DMEM with 10% FBS and gentle shaking. EDTA was then washed away with Tyrode's solution and the cells were resuspended in Tyrode's solution at a concentration of 10^7/ml. All cell culture reagents were purchased from Sigma (Oakville, ON, Canada).

Platelet Aggregation. Washed platelets were pre-incubated for 2 minutes at 37°C in a whole-blood lumi-aggregometer (Chronolog). Platelet aggregation was then initiated by the addition of HT-1080 or A549 cells ($2x10^4$ – $2x10^6$ cells/ml)

and monitored by Aggro-Link software. Platelet aggregation was measured as an extent of light transmittance and then expressed as a percent of maximal stimulus taken at a time point when the maximal stimulus reached 50% transmittance (Jurasz et al. 2001a and b). Platelet samples for flow cytometry were taken at this time point.

Reagents. Prostacyclin (PGI_2), N-Acetyl-Pen-Arg-Gly-Asp-Cys, o-phenanthroline, apyrase, acetylsalicylic acid (aspirin), S-nitroso-n-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) were obtained from Sigma (Oakville, ONT). In some experiments platelets were aggregated in the presence of neutralizing rabbit polyclonal anti-MMP-2 antibodies (Cheung et al. 2000) or control affinity-purified rabbit IgG (Sawicki et al. 1998). These reagents were incubated with platelets for 2 minutes prior to the addition of tumor cells. In experiments where the effects of SNAP and GSNO on tumor cells were examined, these compounds were pre-incubated with the tumor cells for 1 hour at 37 °C. Antibodies were pre-incubated with tumor cells for 2 hours at 37 °C, then washed out of the medium three times with Tyrode's solution. 1H-[1,2,4]Oxadiazole[4,3]quinoxalin-1-one (ODQ) (Alexis; San Diego, CA, USA) an inhibitor of the soluble guanylyl cyclase (Moro et al. 1996) was pre-incubated with the platelets in the aggregometer 5 minutes prior to the addition of SNAP or GSNO.

Fluorescein isothiocyanate (FITC) conjugated monoclonal mouse anti-platelet GP IIb (human CD41) antibodies (isotype IgG1, kappa) and R-

phycoerythrin (RPE) conjugated monoclonal mouse anti-platelet GP Ib (human CD42b) antibodies (isotype IgG2a, kappa) were obtained from DAKO (Mississauga, ON, Canada). PAC-1 FITC monoclonal mouse anti-platelet GP IIb/IIIa antibodies (isotype IgM, kappa) were obtained from Becton Dickinson (San Jose, CA, USA). Von Willebrand Factor coated polystyrene beads (Stewart et al. 1997) were provided by Thrombotics Inc (Edmonton, AB, Canada). The beads were washed and resuspended in an equivalent volume of saline and used for experiments.

Human recombinant TIMP-4 was a generous gift of Dr. Chris Overall from the University of British Columbia.

Matrix Metalloproteinase Assays: Zymography and Reverse Zymography. The aggregates of platelets and tumor cells were centrifuged at 16,000 g at room temperature for 2 minutes yielding the pellet and releasate. The pellets were then homogenized on ice using a Vibra Sonic sonicator (Sonics & Materials Inc. Danbury, CT USA). Both the releasate and the pellet homogenate were then stored at –80°C until assayed for the presence of MMPs by zymography. Zymography was performed using 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with co-polymerized gelatin (2 mg/ml) as previously described (Sawicki et al. 1997 and 1998). Briefly, the SDS-PAGE was run at 4°C for 1-2 hours. After electrophoresis the gels were washed 3 times for 20 minutes in 2% Triton X-100 at room temperature to remove SDS from the gels. Next, the gels were washed 2 times for 20 minutes in Zymography buffer (Tris-HCl pH 7.6

supplemented with 0.15M NaCl, 5 mM $CaCl_2$, and 0.05% NaN_3) at room temperature to remove the Triton X-100. The gels were then incubated in zymography buffer at 37°C for 1-4 days. The gels were then stained with 0.05% Coomassie Brilliant Blue for 1 hour, and then destained in a 4% ethanol and 8% acetic acid solution. Gelatinolytic activity was detected as transparent bands against a blue background of Coomassie Brilliant Blue-stained gelatin. Finally, gelatinolytic activity of bands was quantitated densitometrically.

Reverse zymography was performed as described by Oliver and colleagues (Oliver et al. 1997). Briefly, 12% separating SDS gels were copolymerised with 2 mg/ml gelatin containing 1% SDS and pro-MMP-2 (160 ng/ml). The samples of platelet homogenate (21.5 μg protein) releasate (10 μl), and human recombinant TIMP-1, TIMP-2, TIMP-3 and TIMP-4 standards (0.5 – 5.0 ng) were loaded and subjected to electrophoresis. TIMPs were identified by inhibition of gelatinolysis when compared with standards.

Western Blotting. Platelet and tumor cell homogenates and releasates (5 μg protein) were subject to 12% SDS-PAGE under reducing conditions. Transfer from the SDS-PAGE gel to PVDF membranes (Bio-Rad, Mississauga, Ontario) was performed using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Mississauga, Ontario). TIMP-4 was identified using anti-TIMP-4 rabbit monoclonal antibody (Chemicon, Temecula, Ca). Anti-rabbit peroxidase conjugated polyclonal antibody was obtained from Sigma (Oakville, ONT). Blots were developed using an ECL Plus kit (Amersham, Oakville, Ontario).

Densitometry was performed on protein bands and analyzed using SigmaGel for Windows software (Jandel Corporation, San Rafael, Ca).

Determination of NOS Activity. Nitric oxide synthase (NOS) activity of the HT-1080 and A549 cell lines was determined using the L-citrulline assay, as described by Radomski et al. 1993. Briefly, the rates of NO formation were determined by measuring the rate of conversion of ^{14}C-L-arginine to ^{14}C-L-citrulline. The dependence of L-arginine conversion on NOS was further confirmed using L-NMMA, a selective inhibitor of this enzyme. Results are expressed as pmol/min/mg protein.

Flow Cytometry. Platelet flow cytometry was performed using a Becton Dickinson flow cytometer (FACScan) equipped with a 488 nm wavelength argon laser, 525nm and 575 nm band pass filters for the detection of fluorescein isothiocyanate and R-phycoerythrin fluorescence, and with Cell Quest software. Flow cytometry was performed on both single and double stained platelet samples. To account for spectral overlap between fluorescein isothiocyanate and R-phycoerythrin labels compensation was performed in experiments with double stained platelet samples. To minimize the presence of aggregates, samples of platelets (10 μl of suspension) and fluorescent-labelled antibodies (10 μl containing 1 μg of anti-GP IIb and GP Ib antibodies, and 10 μl containing 0.25 μg of PAC-1 antibodies) were diluted 10 fold using physiological saline. Platelets were identified by forward and side scatter signals. Ten to twenty thousand

platelet specific events were initially analysed by the cytometer. Platelet autofluorescence was measured and subtracted from platelet samples stained with antibodies. Platelet FL-1 autofluorescence was on average 5.2±1.5 units compared to 1041.5±480.4 units for FITC-antibody stained platelets, while platelet FL-2 autofluorescence was on average 2.7±0.7 units compared to 515.0±198.7 units for RPE-antibody stained platelets. Autofluorescence accounted for 0.5% of total fluorescence for both FITC- and RPE-stained platelets. Using Cell Quest software, non-activated platelets were gated, and then this gate was used to analyse activated platelets so as not to analyse platelet aggregates and microparticles. Furthermore, the gated whole platelet population was arbitrarily sub-gated into large-, medium-, and small-sized platelet populations (Figure 19) to determine if all platelet populations respond in the same way (Radomski et al. 2001, Jurasz et al. 2001b). The gated subpopulations were then analysed for mean fluorescence.

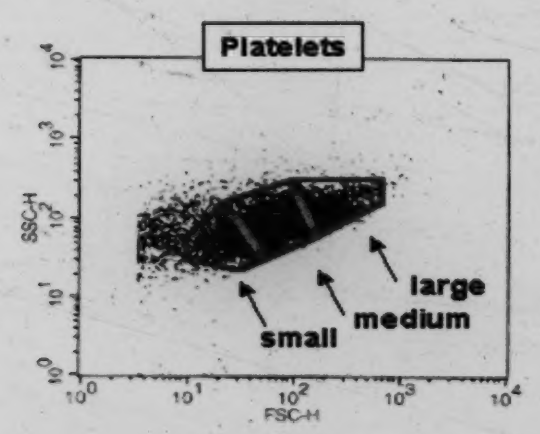

Figure 19. Light scatter dot plot showing gating of platelets into small-, medium-, and large-sized populations.

Microscopy. Tumor cell-platelet samples were viewed using a Nikon phase-contrast microscope equipped with a Nikon camera.

Statistics. Statistics were performed using Prism 3.0 (Graph Pad Software). All means are reported with standard error. One-way analysis of variance (ANOVA), Tukey-Kramer multiple comparisons test, and paired and un-paired Student t-tests were performed where appropriate, and a p-value of less than 0.05 was considered as significant.

Results

MMP-2 in Tumor Cell-induced Platelet Aggregation

Tumor Cell-Induced Platelet Aggregation. HT-1080 and A549 cells were tested for their ability to induce platelet aggregation. When platelets were incubated in the aggregometer for 30 minutes at 37 °C without the addition of tumor cells no platelet aggregation was detected (Figure 20). However, both the HT-1080 and A549 cells induced platelet aggregation in a concentration dependent manner (Figure 20). The EC_{50} for the HT-1080 cells was $0.177 \times 10^6 \pm 0.066 \times 10^6$ cells/ml, while the EC_{50} for the A549 cells was $0.227 \times 10^6 \pm 0.141 \times 10^6$ cells/ml, n=3-7.

Effects of Prostacyclin and N-Acetyl-Pen-Arg-Gly-Asp-Cys on TCIPA. To study if TCIPA induced by HT-1080 cells could be inhibited by classical inhibitors of aggregation, PGI_2, (0.3-30 nM) was pre-incubated for 2 minutes prior to the addition of cancer cells. Figure 21 shows that platelet aggregation induced by maximal effective concentrations of HT-1080 cells ($2x10^5$/ml) was inhibited in a concentration dependent way by PGI_2. Furthermore, N-Acetyl-Pen-Arg-Gly-Asp-Cys, a fibrinogen receptor antagonist (Bogusky et al. 1993), also inhibited aggregation in a concentration dependent manner (Figure 21). The IC_{50} for PGI_2 was 1.00 ± 0.068 nM, while the IC_{50} for N-Acetyl-Pen-Arg-Gly-Asp-Cys was 0.790 ± 0.084 μM, n=3.

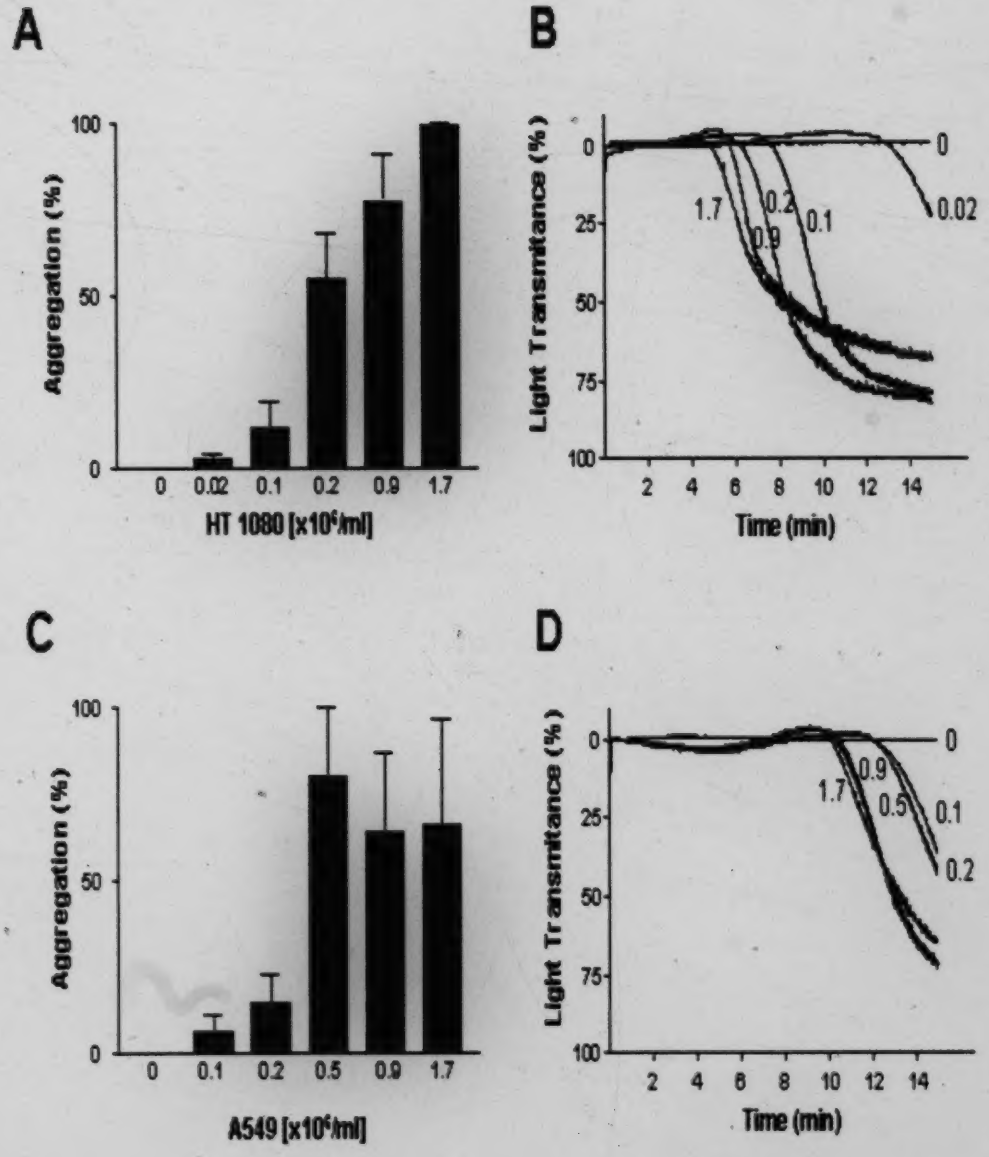

Figure 20. Induction of TCIPA by HT-1080 (A and B) and A549 (C and D) cells. Concentration-effect relationships (A and C) and the corresponding tracings from representative experiments (B and D) describing the aggregatory effects of HT-1080 and A549 cells. Bars are means ± S.E. from 3-7 separate experiments.

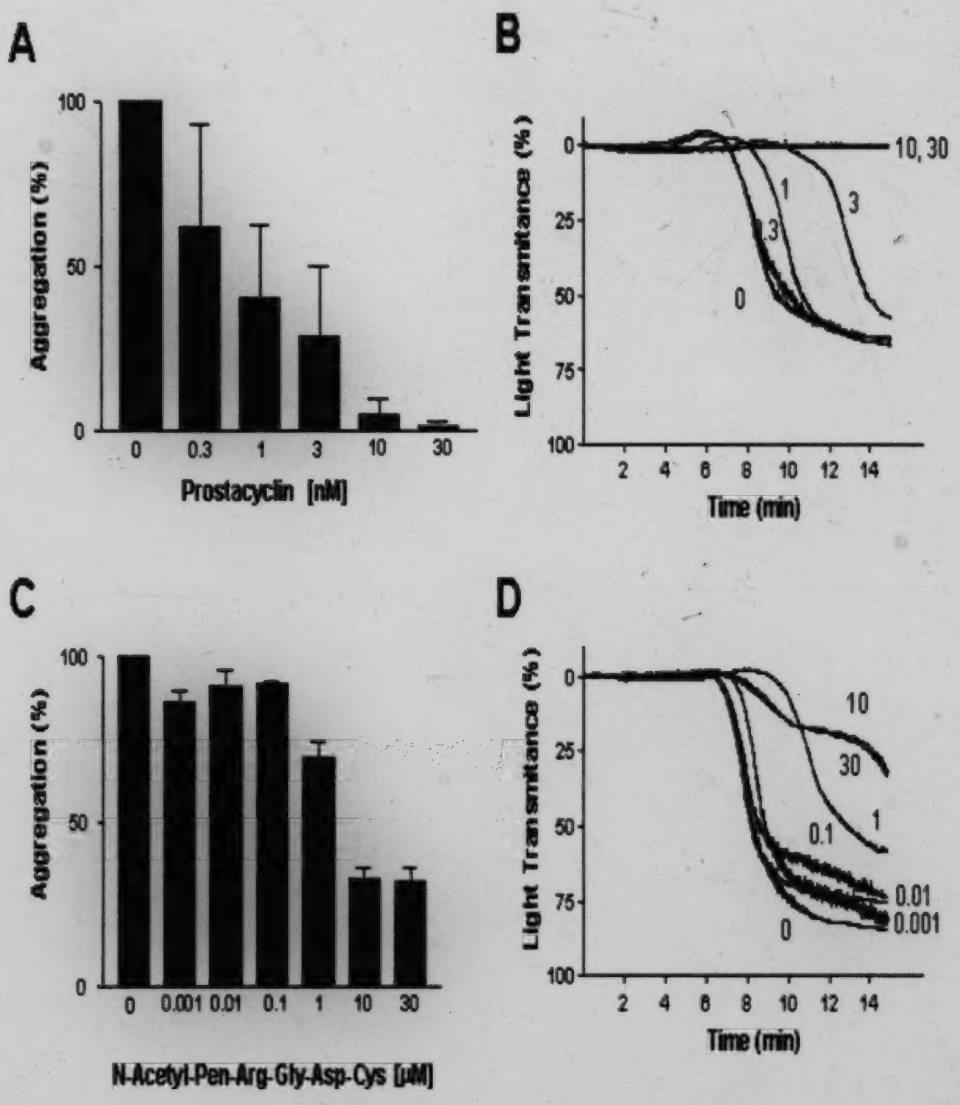

Figure 21. Inhibition of HT-1080 induced TCIPA by prostacyclin (A and B) and N-Acetyl-Pen-Arg-Gly-Asp-Cys (C and D). Concentration-response relationships (A and C) and the corresponding tracings from representative experiments (B and D) describing the inhibitory effects of PGI_2 and N-Acetyl-Pen-Arg-Gly-Asp-Cys. Bars are means ± S.E. from 3 separate experiments.

MMP-2 Activity in Cancer Cell-Platelet Homogenates and their Releasates. To study whether MMP-2 is involved in TCIPA its activity was assayed by zymography. The 72 KDa gelatinase, identified as pro-MMP-2 (Sawicki et al. 1997, Sawicki et al. 1998), was the major gelatinase detected. In the absence of cancer cells the activity of 72 KDa gelatinase in platelets was 158 ± 26 arbitrary units of density/mg protein (n=4) (Figure 22A). As the concentration of HT-1080 cells used to induce platelet aggregation increased (from $2x10^4$ to $5x10^5$ cells/ml), there was a significant ($p<0.0001$, n=4) decrease in the 72 KDa gelatinase activity in platelet-cancer cell homogenates (Figure 22A).

Correspondingly, a significant ($p = 0.0012$, n=4) increase in enzyme activity in the releasate was detected indicating the release of MMP-2 during TCIPA (Figure 22B). However, when aggregation was induced by higher concentrations of HT-1080 ($2x10^6$ cells/ml) there was an increase in 72 KDa gelatinase activity in the homogenate ($p<0.01$, n=4).

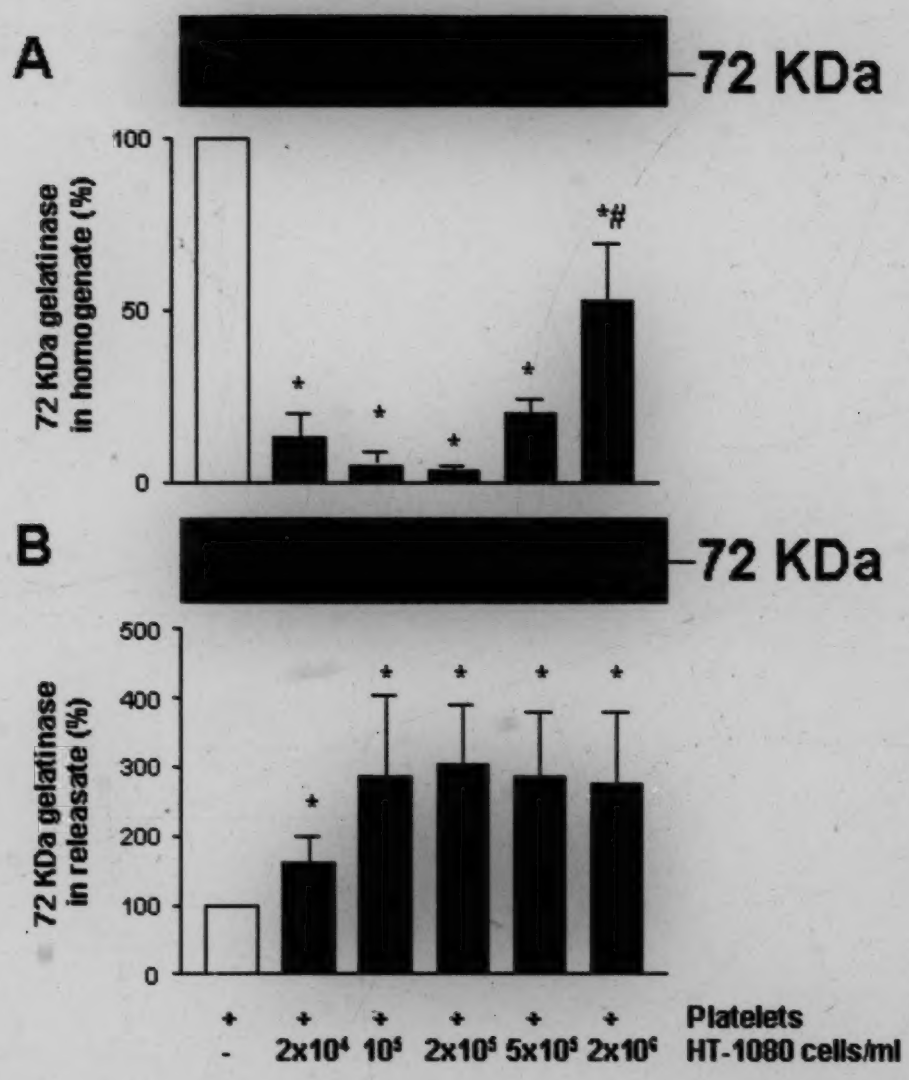

Figure 22. 72 KDa gelatinase activity in the homogenates (A) and releasates (B) of HT-1080 cells and platelets. Insets show representative zymograms. Bars are means ± S.E. from 4 separate experiments. * $p<0.05$ platelets vs. platelets aggregated with HT-1080 cells. # $p<0.05$ platelets aggregated with 10^6 HT-1080 cells vs. platelets aggregated with 10^4 HT-1080 cells. + and - denotes the presence or absence of treatments.

Release of MMP-2 from Tumor Cells. The activity of 72 KDa gelatinase released during incubation of HT-1080 cells and A549 cells was assayed. Equal numbers of HT-1080 and A549 cells (10^7 cells/ml) were incubated for 1 hour at 37 °C and the activity of 72 KDa gelatinase was measured by zymography. Under these conditions HT-1080 cells secreted significantly more 72 KDa gelatinase than A549 cells ($p < 0.0001$, n=3) (Figure 23A).

Release of 72 KDa Gelatinase Induced by HT-1080 and A549 Cells and its Effects on Aggregation. Platelet aggregation was induced by the same number of HT-1080 and A549 cells (10^5 cells/ml). HT-1080 cells resulted in the release of greater amounts ($p=0.012$, n=4) of 72 KDa gelatinase than A549 cells during TCIPA (Figure 23B). Finally, the aggregatory effect of HT-1080 cells was significantly greater than that induced by A549 cells (Figure 23C).

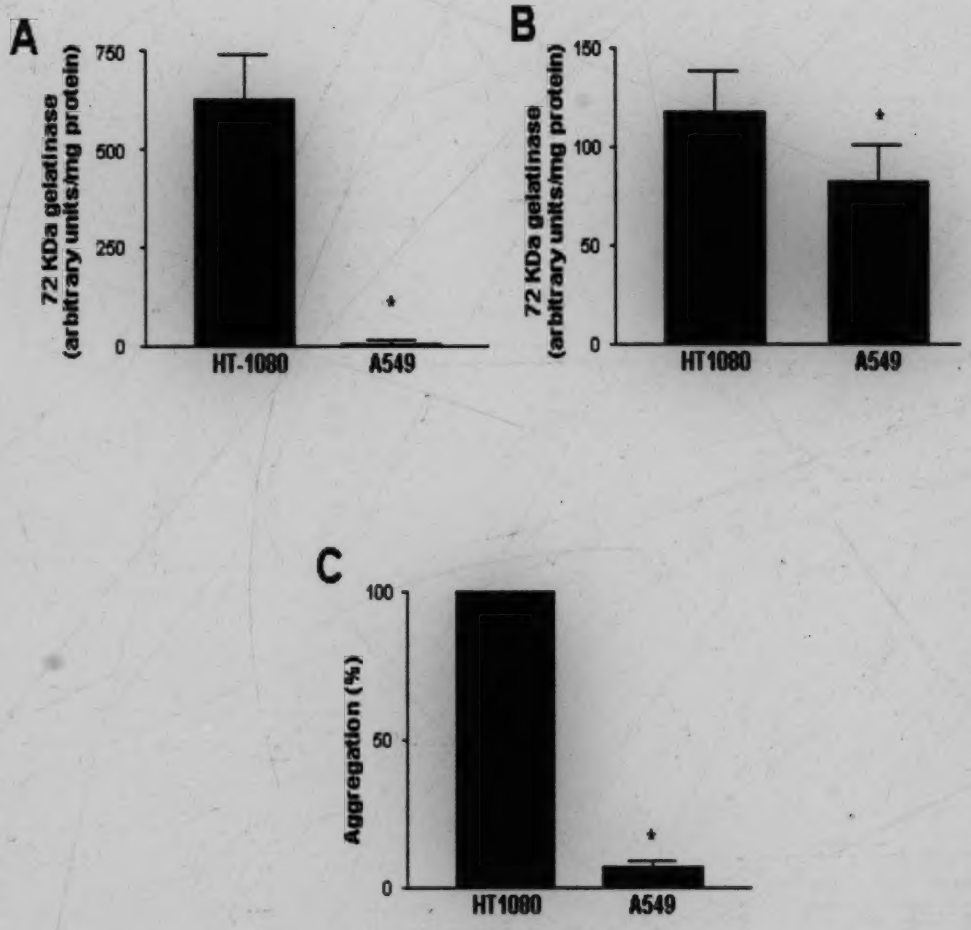

Figure 23. Comparison of 72 KDa gelatinase release from HT-1080 and A549 cells and their effects on TCIPA. The release of 72 KDa gelatinase from tumor cells (A), the enzyme release during TCIPA (B), and the corresponding effects on aggregation (C). Bars are means ± S.E. from 3-4 separate experiments. * $p<0.05$ A549 vs. HT-1080 cells.

Effects of MMP Inhibition on TCIPA. Incubation of platelets with neutralizing anti-MMP-2 antibody (Cheung et al. 2000), but not with control IgG (each at 10 μg/ml), resulted in a significant ($p=0.03$, $n=3$) inhibition of aggregation induced by HT-1080 cells (Figure 24A). Furthermore, pre-incubation of HT-1080 cells for 2 hours at 37 °C with anti-MMP-2 antibody, but not with control IgG (each 10 μg/ml), significantly ($p=0.0195$, $n=3$) reduced the aggregating effects of cancer cells (Figure 24B) indicating that MMP-2 expressed by cancer cells contributes to TCIPA. Moreover, a synthetic inhibitor of MMPs, phenanthroline (1--1000 μM) (Sawicki et al. 1997), inhibited aggregation induced by HT-1080 cells in a concentration dependant manner (Figure 24C).

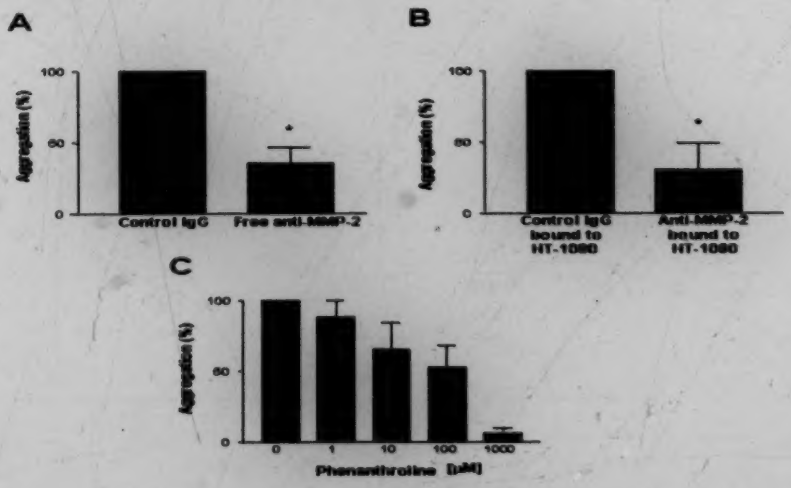

Figure 24. Inhibition of TCIPA by neutralizing anti-MMP-2 antibody (A and B) and phenanthroline (C). Anti-MMP-2 antibody (10 μg/ml), or IgG control (10 μg/ml), was either pre-incubated with platelets (A) or pre-incubated with HT-1080 cells (B) prior to HT-1080-induced aggregation. Concentration-response relationship to phenanthroline (C). TCIPA was induced by HT-1080 cells ($2x10^5$-$4x10^5$ cells/ml). Bars are means ± S.E. from 3 separate experiments. *$p<0.05$ anti-MMP-2 antibody vs. control.

Contributions of Thromboxane-, ADP-, and MMP-2- Dependant Pathways of Aggregation to TCIPA. To test the relative contributions of the different platelet aggregating pathways to TCIPA induced by the HT-1080 cells we used inhibitors of three major platelet-aggregating pathways (Sawicki et al. 1997). To inhibit thromboxane A_2-, MMP-2-, and ADP-mediated pathways of aggregation acetylsalicylic acid (aspirin 100 μM), phenanthroline (100 μM) and apyrase (250 μg/ml) were used, respectively. Combined inhibitory effects of these compounds abolished TCIPA induced by HT-1080 cells (Figure 25).

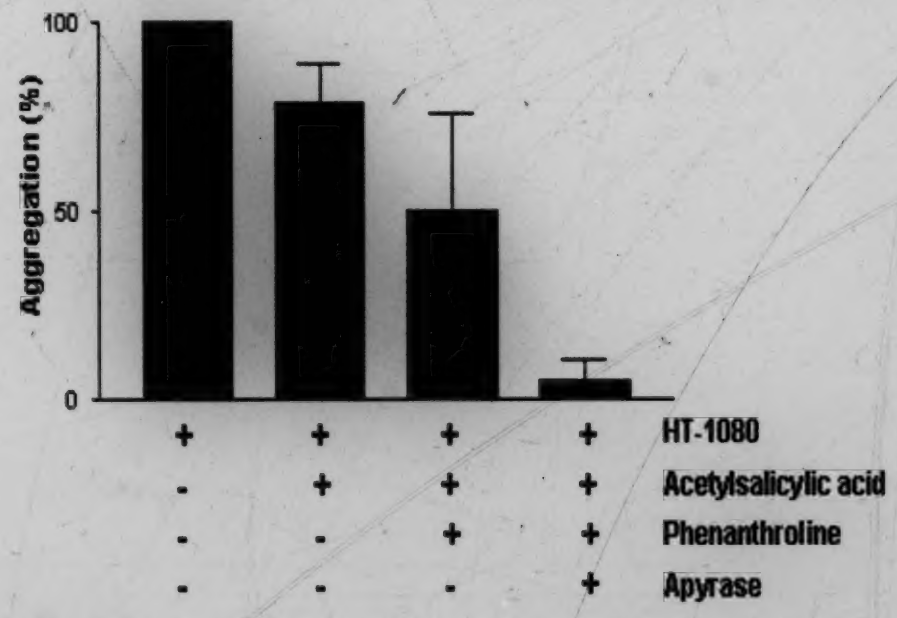

Figure 25. Inhibition of platelet aggregation induced by HT-1080 cells (10^5/ml) by acetylsalicylic acid (100μM), phenanthroline (100μM), and apyrase (250μg/ml). Bars are means ± S.E. from 3 separate experiments. + and - denotes the presence or absence of treatments, respectively.

Effects of SNAP and GSNO on Aggregation and MMP-2 Release during TCIPA. Since NO is a potent inhibitor of platelet activation and aggregation (Radomski et al. 1987c, Radomski et al. 1987d), we tested the effects of SNAP and GSNO (each at 0.01–100 μM) on TCIPA induced by HT-1080 cells. Both SNAP and GSNO inhibited TCIPA, as shown by aggregometry (Figure 26) and phase-contrast microscopy (Figure 27). The IC_{50} for SNAP was 1.34 ± 0.67 μM, while the IC_{50} for GSNO was 0.251 ± 0.125 μM, n=8. Moreover, inhibition of TCIPA by SNAP (100 μM) and GSNO (100 μM) was accompanied by a significant reduction ($P=0.0154$, n=5, and $P<0.0001$, n=5, respectively) in the release of 72 KDa gelatinase during TCIPA (Figure 28A). Finally in the absence of platelets, both SNAP and GSNO (100 μM) inhibited ($P=0.0196$, n=12, and $P=0.0037$, n=12, respectively) the release of 72 KDa gelatinase from HT-1080 cells after being incubated with HT-1080 cells for one hour at 37°C (Figure 28B).

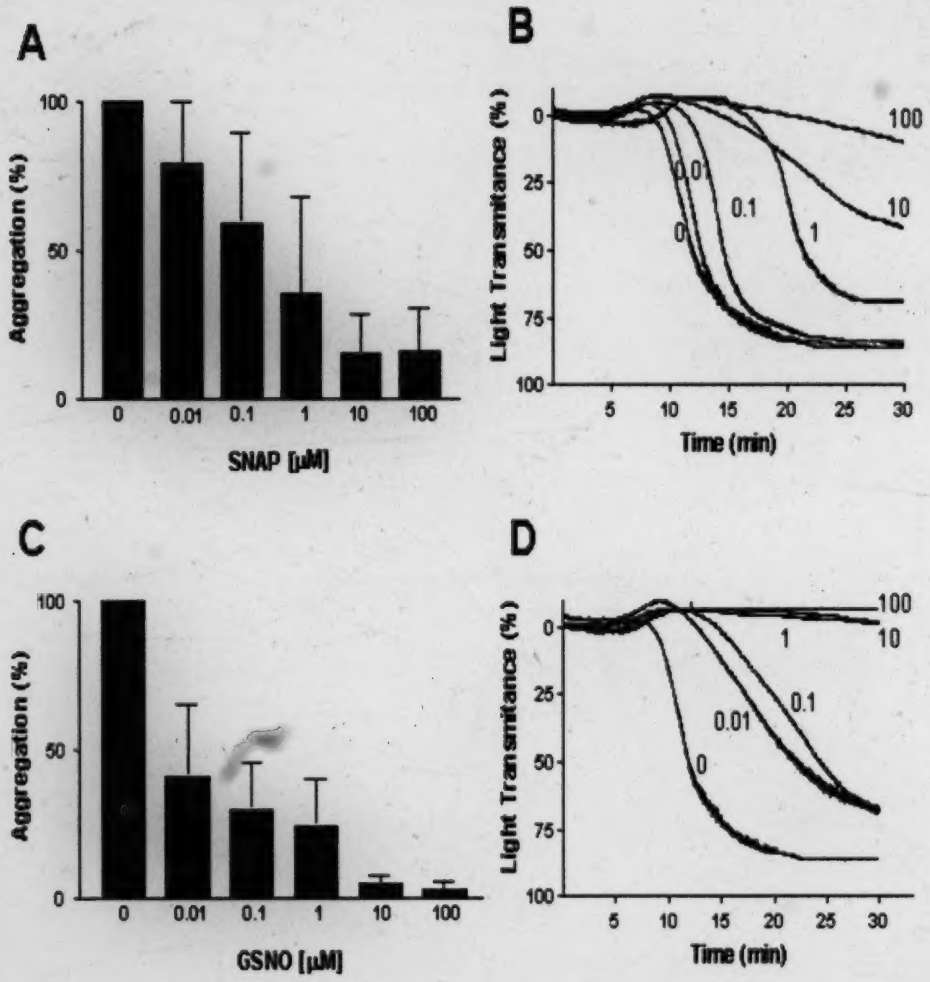

Figure 26. Inhibition of HT-1080-induced TCIPA (1-4x10^5 cells/ml) by SNAP (A and B) and GSNO (C and D). Concentration-response relationships (A and C) and the corresponding tracings for representative experiments (B and D) describing the inhibitory effects of SNAP and GSNO. Bars are means ± S.E. from 5-8 separate experiments.

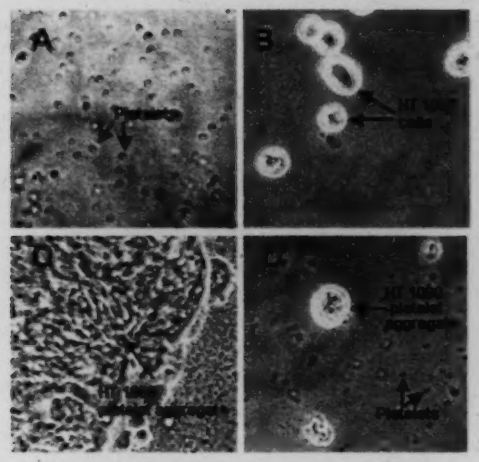

Figure 27. Phase-contrast microscopy of TCIPA and its inhibition with GSNO (1 μM). (A) Platelets. (B) HT-1080 cells. (C) TCIPA. (D) Effect of GSNO. (A), (B), (C) and (D): x400.

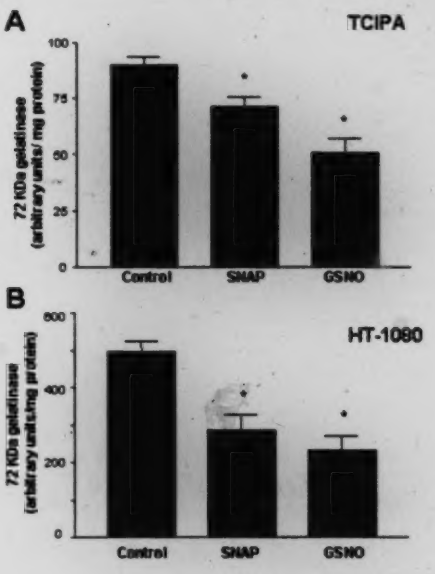

Figure 28. Inhibition of the release of 72 KDa gelatinase by SNAP (100 μM) and GSNO (100 μM) during HT-1080 induced TCIPA ($3.5x10^5$ cells/ml) (A) and from HT-1080 cells in culture (B). Bars are means ± S.E. from 5-12 separate experiments. *$p<0.05$ SNAP or GSNO vs. control.

Effects of ODQ on TCIPA and MMP-2 Release during Inhibition of Aggregation Induced by SNAP and GSNO. Finally, we investigated whether the inhibitory effects of NO on MMP-2 release during TCIPA were dependant on the production of cyclic GMP. ODQ, a selective inhibitor of GC-S (Moro et al. 1996), reversed the inhibition of TCIPA by SNAP (Figure 29A and B). Moreover, ODQ (30 μM) also significantly reversed the decrease in 72 KDa gelatinase release caused by SNAP and GSNO (both at 100 μM) (p= 0.0359, n=3, and p = 0.0032, n=4, respectively) during TCIPA (Figure 29C and D).

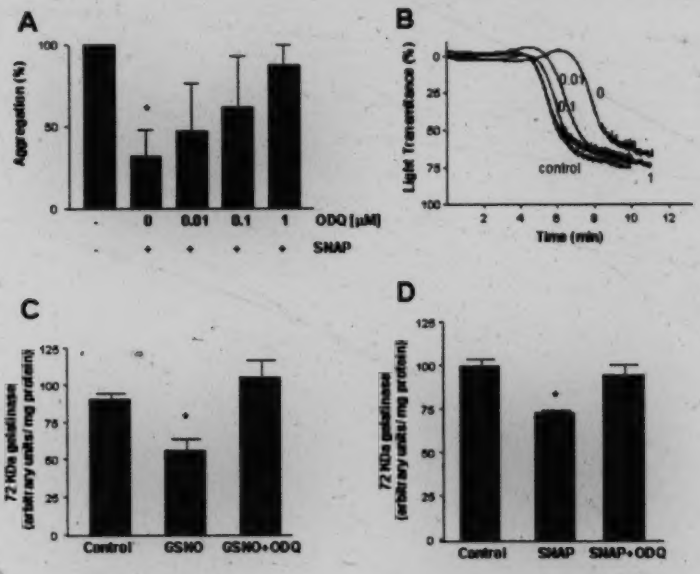

Figure 29. Reversal by ODQ of inhibitory effects of GSNO and SNAP on TCIPA. (A) Concentration-dependant reversal of the inhibitory effects of SNAP (1μM) by ODQ and the corresponding tracings (B) from representative experiments. Reversal by ODQ of inhibition of 72 KDa gelatinase release during TCIPA by GSNO (100 μM) (C) and SNAP (100 μM) (D). TCIPA was induced by HT-1080 (10^5-10^6 cells/ml). Bars are means ± S.E. from 4 separate experiments. + and - Denotes the presence or absence of treatments, respectively. * $p<0.05$ treatments vs. control.

NOS Activity of HT-1080 and A549 Cancer Cells. The ability to convert L-arg to NO and L-citrulline was determined for the two cancer cell lines. Total NOS activity was determined and found to be significantly greater (p=0.0127, n=4) for A549 cells (25.7±6.2 pmol/min/mg protein) than HT-1080 cells (14.1±3.7 pmol/min/mg protein) (Figure 30).

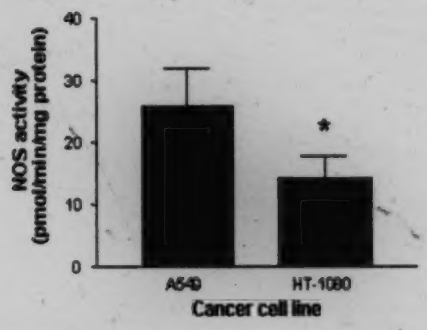

Figure 30. Total NOS activity in A549 and HT-1080 cells. Bars are means ± S.E. from 4 separate experiments. * p<0.05 HT-1080 vs. A549.

Effects of NO and Prostacyclin on Platelet Receptors during TCIPA

TCIPA in the Presence of sVWF. Incubation of HT-1080 cells with platelets resulted in TCIPA (Figure 31). This effect was potentiated in the presence of sVWF (Figure 31 A-C), as evidenced by the faster onset of aggregation as compared to HT-1080 cells alone (p=0.0117, n=9). Furthermore, TCIPA in the presence of sVWF was two-phased. The first phase marked platelet adhesion to the immobilized sVWF and the second phase platelet-platelet-tumor cell aggregation (Figure 31A and B).

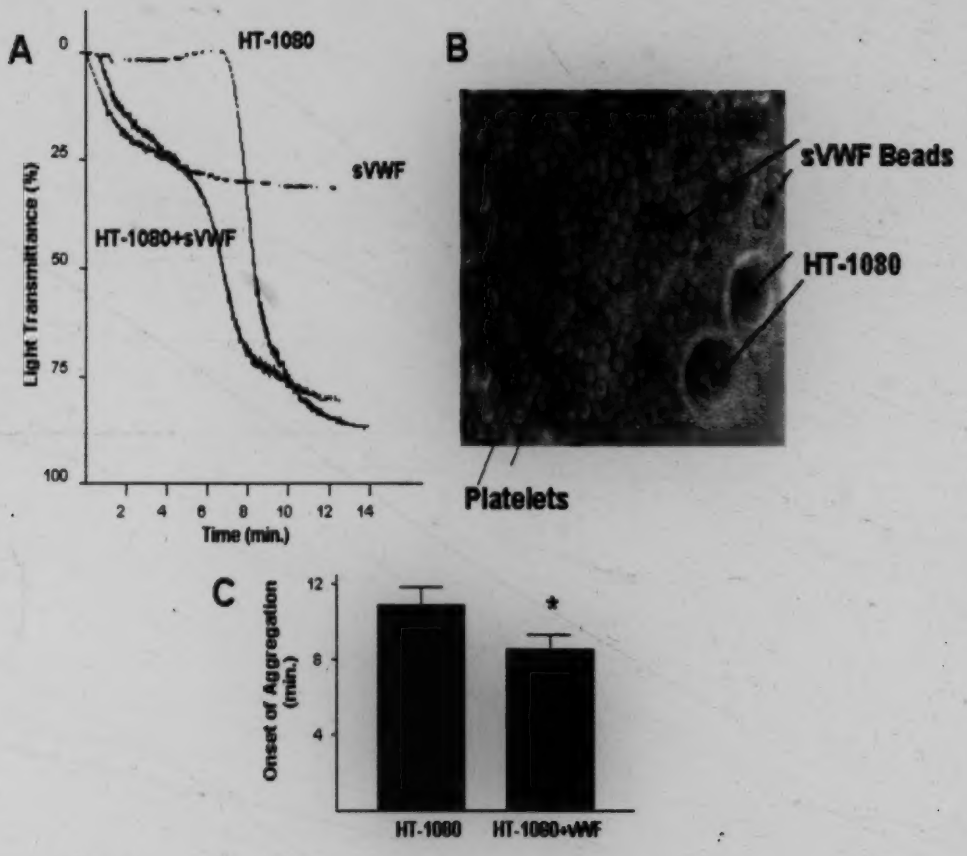

Figure 31. (A) Representative traces showing TCIPA in the presence or absence of sVWF (1.6 μg/ml). TCIPA was induced by HT-1080 cells ($5x10^3$ cells/ml). (B) Phase-contrast microscopy of HT-1080 induced TCIPA in the presence of sVWF, x400. (C) Onset of platelet aggregation induced by HT-1080 cells ($5x10^3$ cells/ml) versus HT-1080 cells in the presence of sVWF (1.6 μg/ml). Bars are means ± S.E. from 9 separate experiments. *, $p<0.05$, HT-1080 versus HT-1080 and sVWF.

Effects of GSNO and PGI_2. GSNO (100 μM) and PGI_2 (30 nM) both significantly ($p<0.01$ and $p< 0.001$, respectively; n=6) inhibited HT-1080 induced platelet aggregation (Figure 32A and B).

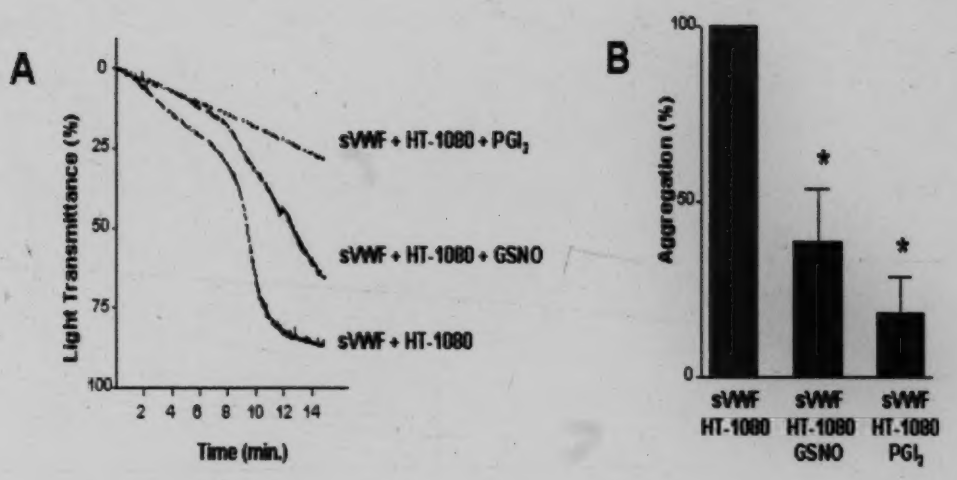

Figure 32. Representative traces (A) and the statistical analysis (B) showing the effects of GSNO (100 μM) and PGI_2 (30 nM) on TCIPA in the presence of sVWF (1.6 μg/ml). TCIPA was induced by HT-1080 cells ($5x10^3$ cells/ml). Bars are means ± S.E. from 6 separate experiments. *, $p<0.05$, treatments versus control.

GP Ib and GP IIb/IIIa during the Adhesion Phase of TCIPA. In the absence of HT-1080 cells, sVWF caused an increase in the surface expression of platelet GPIb, as seen by a significant ($p=0.0283$; n=4) increase in mean fluorescence when measured by flow cytometry (Figure 33A). However, during the platelet adhesion phase of TCIPA in the presence of sVWF, HT-1080 cells caused a decrease in the platelet surface expression of GPIb, as compared to sVWF

alone. This was evident by the significant (p=0.0231, n=4) decrease in mean fluorescence (Figure 33B). No significant changes (p>0.05, n=3) in the surface expression of GP IIb/IIIa were detected during these experiments.

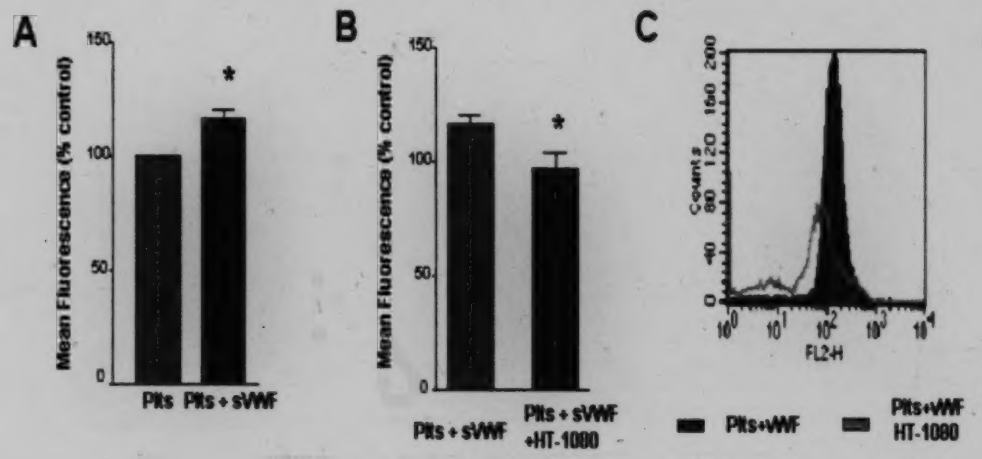

Figure 33. Effects of sVWF (1.6 μg/ml) on platelet surface expression of GPIb in the absence (A) or presence (B) of HT-1080 cells. Histogram showing the expression of GP Ib (C). TCIPA was induced by HT-1080 cells ($5x10^3$ cells/ml). Bars are means ± S.E. from 4 separate experiments. *, p<0.05, treatment versus control. Plts, platelet suspensions.

GP IIb/IIIa and GP Ib during the Aggregation Phase of TCIPA. During the aggregation phase of TCIPA in the presence of sVWF GP IIb/IIIa expression significantly (p<0.01, n=4) increased (Figure 34A). Similar, during TCIPA in the absence of sVWF there was a significant (p=0.0010, n=9) increase in the platelet surface expression of GP IIb/IIIa (Figure 35). Platelet activation with sVWF in the absence of HT-1080 cells did not cause any changes in GP IIb/IIIa (Figure 34A). TCIPA in the presence of sVWF resulted in a significant (p<0.001, n=4) decrease in platelet surface GP Ib (Figure 34B). TCIPA in the absence of sVWF also led to

a significant (p=0.0001, n=9) decrease in platelet surface expression of GP Ib (Figure 35).

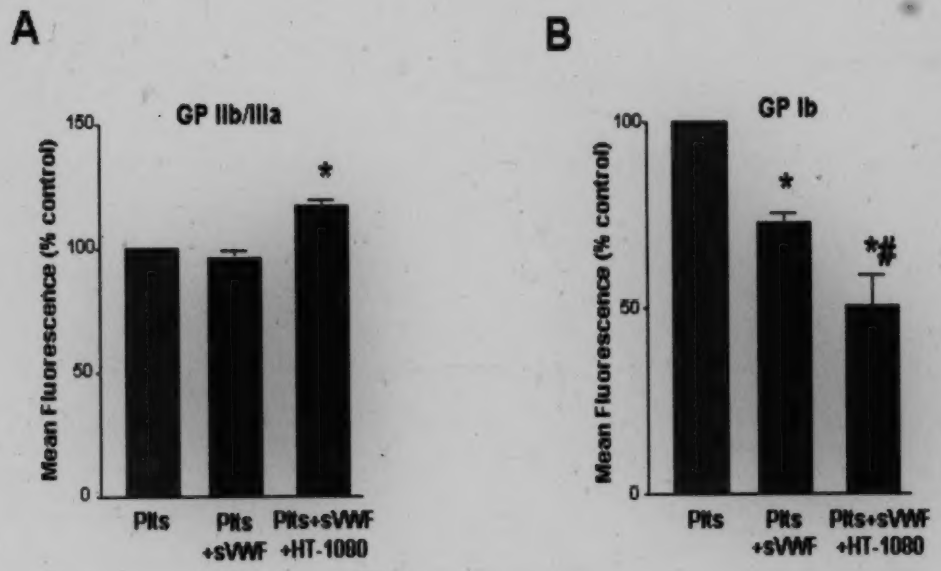

Figure 34. Effects of HT-1080 cells on GP IIb/IIIa and GP Ib expression during the aggregation phase of TCIPA (5x 10^3 cells/ml) in the presence of sVWF (1.6 μg/ml). Bars are means ± S.E. from 4 separate experiments. *, $p<0.05$, treatments versus control. #, $p<0.05$ Platelets and VWF versus platelets and VWF and HT-1080 cells.

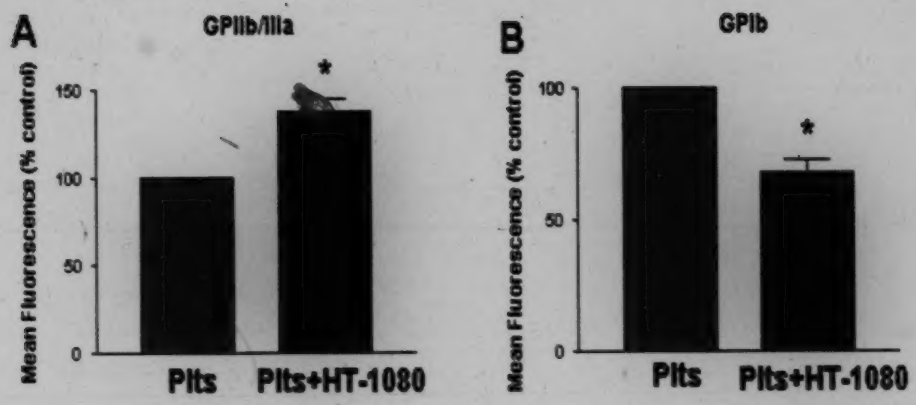

Figure 35. Effects of HT-1080 cells on GP IIb/IIIa and GP Ib expression during TCIPA (5×10^3 cells/ml) in the absence of sVWF. Bars are means ± S.E. from 9 separate experiments. *, $p < 0.05$ Treatment versus control.

Effects of GSNO and PGI_2 on Total (non-activated and activated) GPIIb/IIIa.

Flow cytometry performed on platelets pre-incubated with GSNO (100 μM) or PGI_2 (30 nM) and then activated by HT-1080 cells and sVWF was analysed by examining different sized platelet populations. PGI_2, but not GSNO, in large, medium and small platelet subpopulations significantly ($p<0.01$, $p< 0.05$ and $p<0.01$, respectively, n=6) inhibited HT-1080-mediated increase in the surface number of platelet GP IIb/IIIa (Figure 36A, B, and C). In contrast, GSNO failed to prevent the increase in platelet surface GP IIb/IIIa ($p>0.05$; n= 6) (Figure 36A, B and C).

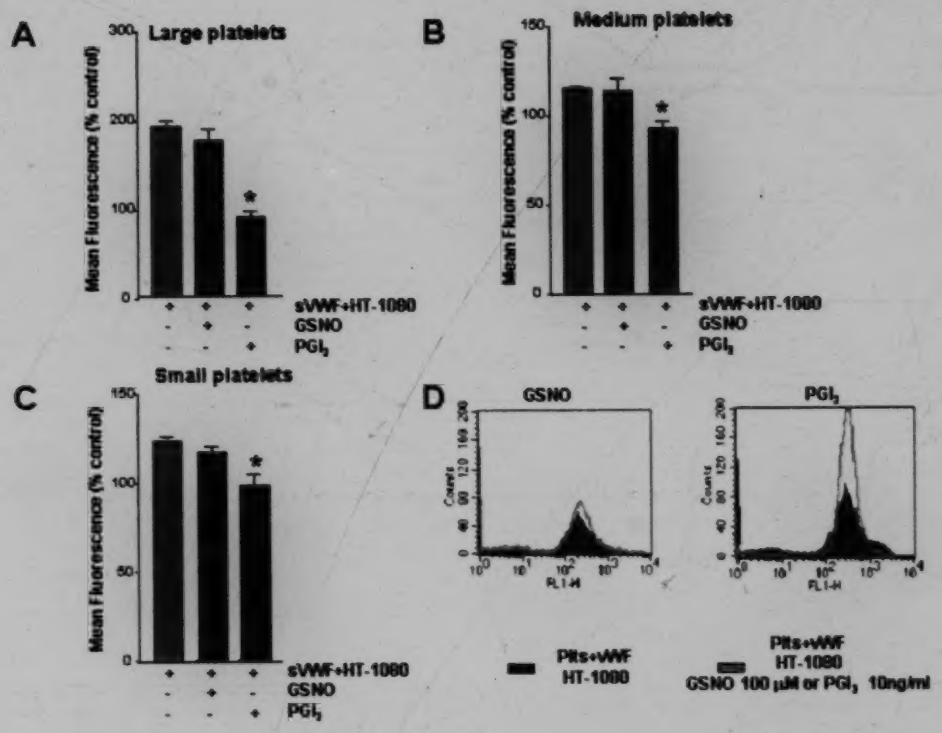

Figure 36. Effects of GSNO (100 μM) and PGI_2 (30 nM) on expression of platelet GP IIb/IIIa in different-sized platelet populations during TCIPA. (A) large platelets. (B) medium platelets. (C) small platelets. (D) Representative histogram of medium sized platelets.TCIPA was induced by HT-1080 cells ($5x10^3$ cells/ml) and sVWF (1.6 μg/ml). Bars are means ± S.E. from 6 separate experiments. *, $p<0.05$, treatments versus control. + and - denote the presence or absence of treatments, respectively.

Furthermore, during TCIPA in the absence of sVWF GSNO did not decrease the surface number of GP IIb/IIIa; however, GSNO did, in fact, inhibit TCIPA (Figure 37).

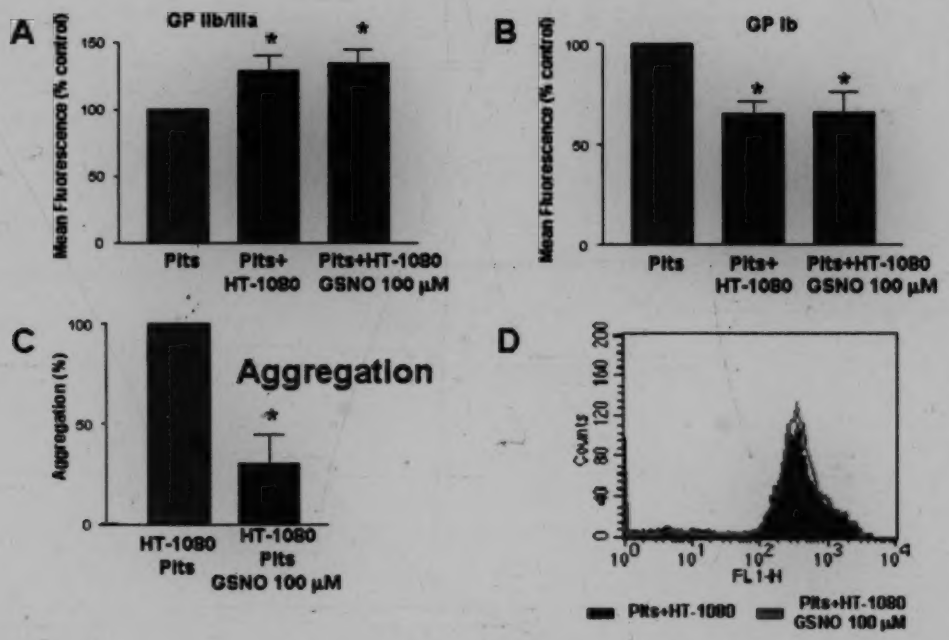

Figure 37. Effect of GSNO (100 µM) on expression of platelet GP IIb/IIIa (A) and GP Ib (B) during TCIPA measured as mean fluorescence. Effect of GSNO (100 µM) on TCIPA (C). Histogram of GP IIb/IIIa expression. TCIPA was induced by HT-1080 cells (5 x 10^3 cells/ml). Bars are means ± S.E. from 6 separate experiments. *, $p < 0.05$ Treatment versus control. Plts, platelet suspensions.

Effects of GSNO on activated GP IIb/IIIa. Flow cytometry performed with the PAC-1 antibody that recognizes only the activated GP IIb/IIIa showed that in large, medium, and small platelet subpopulations GSNO (100 µM) significantly ($p=0.0103$, $p = 0.0176$, and $p=0.0110$, respectively, $n=3$) inhibited the activation of GP IIb/IIIa during TCIPA (Figure 38A, B, and C). GSNO (100 µM) also caused inhibition of activation of GPIIb/IIIa during TCIPA in the absence of vWF, as seen by the significant ($p=0.0284$, $n=6$) decrease in mean fluorescence (Figure 39).

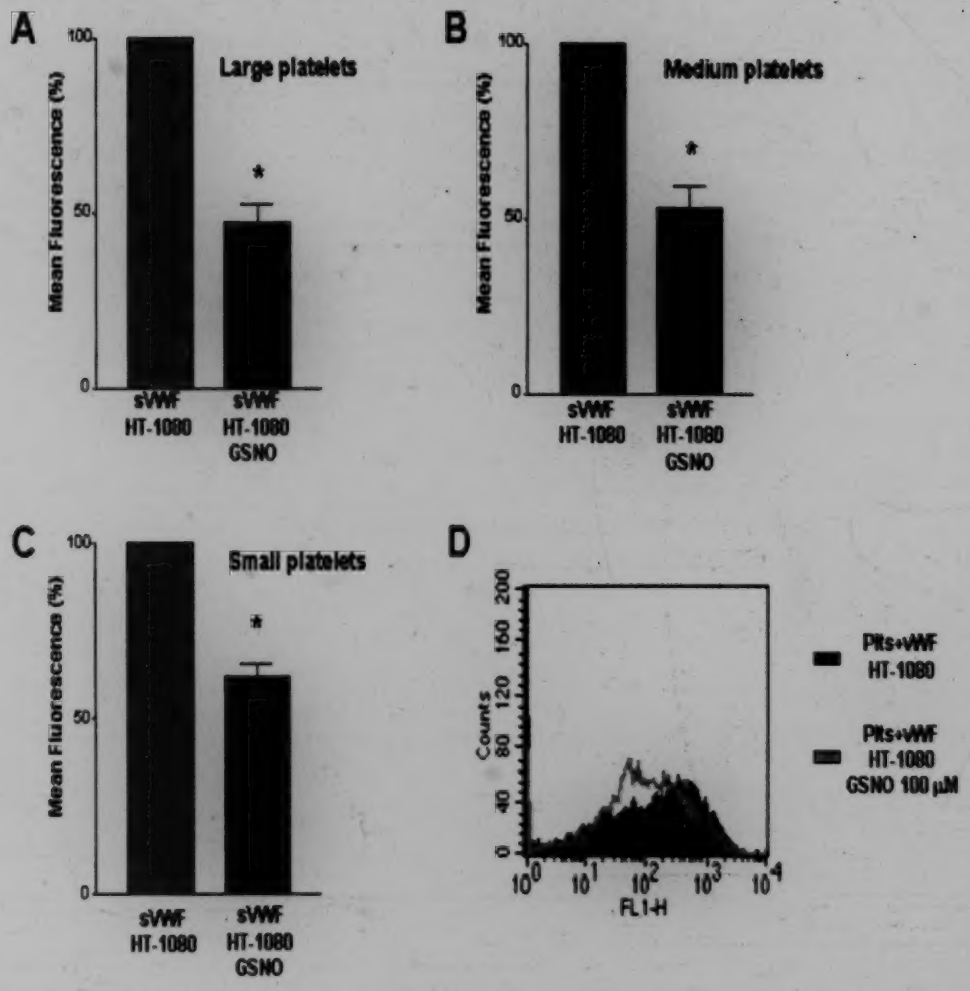

Figure 38. Effects of GSNO (100 μM) on the activation of platelet GP IIb/IIIa during TCIPA in the presence of sVWF measured with the PAC-1 antibody. TCIPA was induced by HT-1080 cells ($5x10^3$ cells/ml) and sVWF (1.6 μg/ml). Bars are means ± S.E. from 3 separate experiments. *, $p<0.05$, treatment versus control.

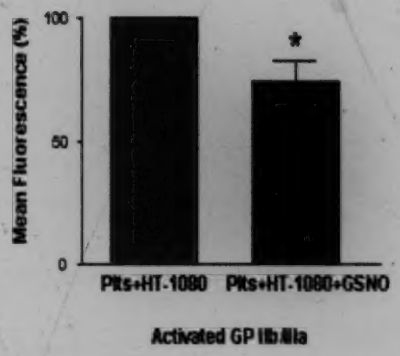

Figure 39. Effect of GSNO (100 μM) on the activation of platelet GP IIb/IIIa during TCIPA measured as mean fluorescence with the PAC-1 antibody. TCIPA was induced by HT-1080 cells (5×10^3 cells/ml). Bars are means ± S.E. from 6 separate experiments. *, $p < 0.05$ Treatment versus control. Plts, platelet suspensions.

TIMP-4 in TCIPA

Role of TIMP-4 in TCIPA. We have identified TIMP-4 as the major TIMP in human platelets (Figure 40). Therefore the effects of TIMP-4 were studied during TCIPA. Both HT-1080 cells and platelets were found to produce TIMP-4 (Figure 41). In addition, both cell types released basal levels of TIMP-4 when incubated in the aggregometer alone. Upon TCIPA by the HT-1080 cells the level of released immunoreactive TIMP-4 did not increase ($p > 0.05$; n=5). This is in stark contrast to platelet aggregation induced by a traditional agonist, such as collagen, which resulted in the release of TIMP-4 from platelets (Figure 42).

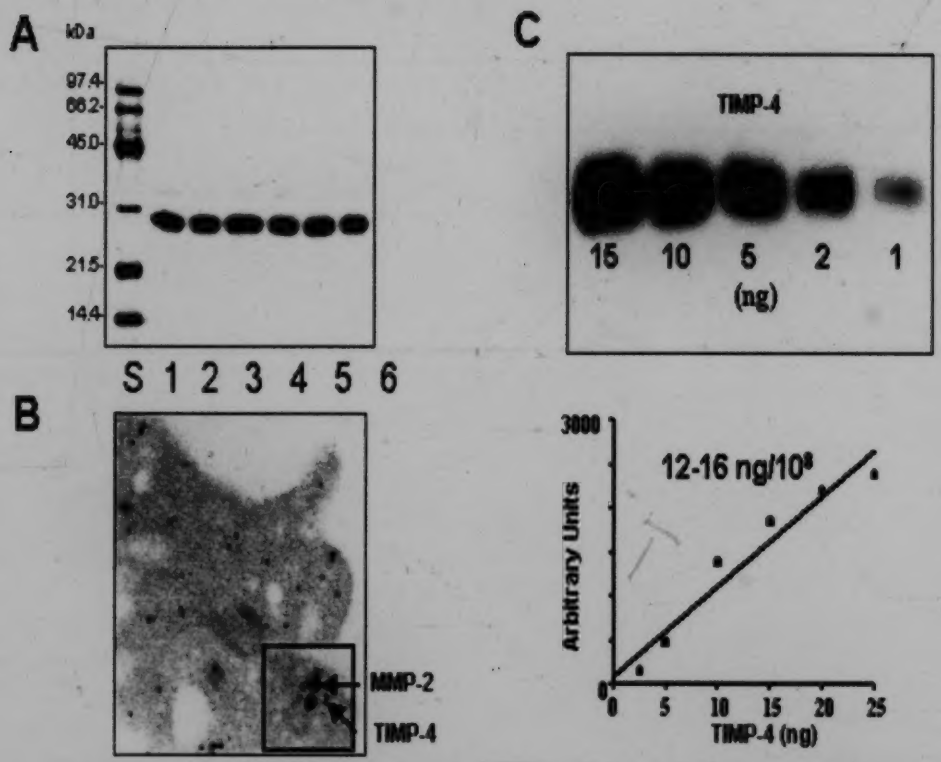

Figure 40. Western blot analysis from six blood donors (A) and electron microscopy (B) identifying TIMP-4 as the major TIMP found in platelets. 12-16 ng of TIMP-4 is found in 10^8 platelets. These experiments were performed in collaboration with Dr. Anna Radomski.

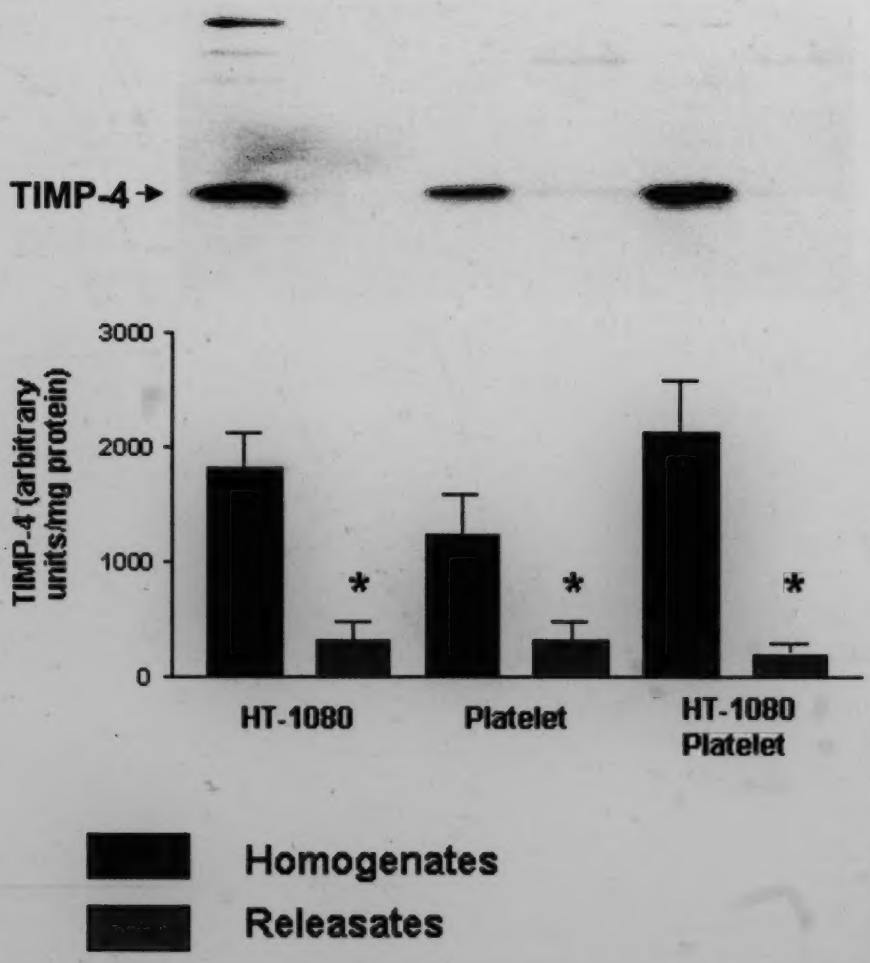

Figure 41. Western blot analysis of TIMP-4 in homogenates of HT-1080 cells and platelets (black bars) and their respective releasates (gray bars) during TCIPA. Insert shows a representative blot. Bars are means ± S.E. from 4 separate experiments. *, $p<0.05$, Releasates versus homogenates.

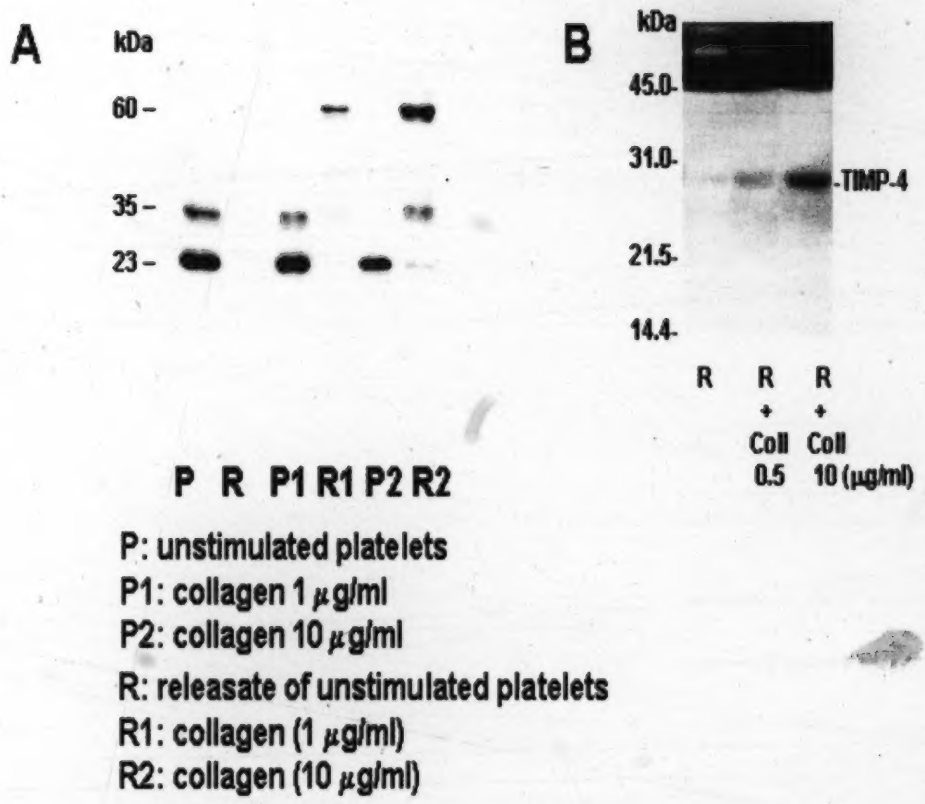

Figure 42. Western blot analysis (A) and reverse zymography (B) analysis showing the release of TIMP-4 from platelets when stimulated with collagen. These experiments were performed in collaboration with Dr. Anna Radomski.

Effects of Recombinant TIMP-4 on TCIPA. Pre-incubation of platelets with 5 ng/ml and 50 ng/ml recombinant TIMP-4 resulted in a reduction of TCIPA to 84±10% (n=3) and 28±12% (n=3) of control, respectively (Figure 42). TIMP-4 at 500 ng/ml exerted no inhibitory effect ($p > 0.05$; n=3). Thus, pre-incubation of platelets with rTIMP-4 (5-500 ng/ml) resulted in a biphasic concentration-response.

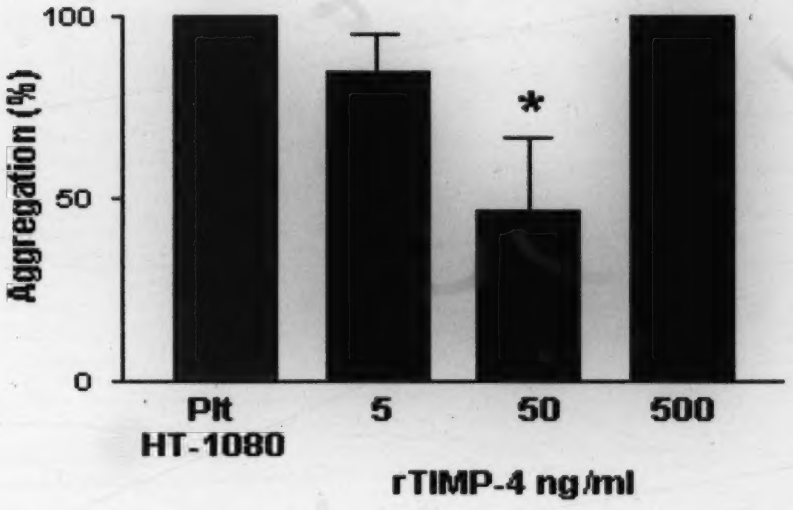

Figure 43. Effect of recombinant TIMP-4 on TCIPA. TCIPA was induced by HT-1080 cells (5 x 10^3 cells/ml). Bars are means ± S.E. from 4 separate experiments. *, $p<0.05$, treatment versus control.

Discussion

Experimental Approach

The objective of my investigation was to study the role of MMPs in TCIPA, focusing on a novel mediator of platelet aggregation MMP-2 (Sawicki et al. 1997). Furthermore, I investigated the effects of NO, a known inhibitor of platelet aggregation (Radomski et al. 1987c and d) and TCIPA (Radomski et al. 1991), on the release and the biological activity of MMP-2. In addition, I investigated the effects of both NO and PGI_2 on the expression of platelet GP IIb/IIIa and Ib receptors during aggregation by tumor cells. Moreover, these experiments were carried out in a new model of TCIPA that incorporated a novel platelet agonist, solid-phase vWF. This new model allowed me to study both platelet aggregation and platelet adhesion during stimulation by tumor cells. Finally, the discovery of TIMP-4 as the major TIMP present in platelets led to the study of its role in TCIPA.

Role and Regulation of MMP-2 in TCIPA

Two human tumor cell lines were used: HT-1080 fibrosarcoma and A549 lung epithelial carcinoma cells in order to study the role of MMP-2 in TCIPA. HT-1080 cells were found to be more potent inducers of platelet aggregation than A549 cells. The fact that the A549 cells are not potent stimulators of platelet aggregation is consistent with the results obtained by Heinmoller et al. 1996. Furthermore, it was found that HT-1080 cells secreted more MMP-2 than the A549 cells. Therefore, the majority of the remaining experiments studying the

biological role of MMP-2 in TCIPA and its interactions with NO were carried out using HT-1080 cells.

First, it was shown that the interactions between HT-1080 cells and platelets led to aggregation, as evidenced by phase-contrast microscopy and the inhibition of TCIPA by PGI_2. Prostacyclin is the most potent known inhibitor of platelet aggregation (Moncada et al. 1976). Moreover, PGI_2 is known to inhibit TCIPA induced by other tumor cell lines (Honn et al. 1981).

Having established that the interactions between HT-1080 cells and platelets result in aggregation, the role of MMP-2 in TCIPA was studied. It was found that aggregation induced by HT-1080 cells was associated with the release of 72 KDa gelatinase suggesting that MMP-2, in addition to agonist-induced aggregation (Sawicki et al. 1997 and 1998), contributes to TCIPA. Several lines of evidence support this hypothesis. First, the release of this enzyme during TCIPA induced by HT-1080 cells was concentration-dependant. Second, phenanthroline, an MMP inhibitor that inhibits agonist-induced platelet aggregation (Sawicki et al. 1997), reduced TCIPA. Third, TCIPA was significantly decreased in the presence of neutralizing anti-MMP-2 antibody, but not by control IgG. Moreover, the HT-1080 cells that had been pre-incubated for 2 hours with this antibody, but not with control IgG, showed a decreased ability to induce TCIPA. Since in these experiments anti-MMP-2 antibody was washed out of the medium of HT-1080 cells, its aggregation-inhibitory effects are clearly associated with the neutralization of MMP-2 expressed at the surface of HT-1080

cell membrane. Thus, both platelets and cancer cells may contribute to the MMP-2 pool involved in TCIPA.

The mechanism(s) of the pro-aggregating actions of MMP-2 are being now elucidated. It has recently been shown that gelatinase B (MMP-9) may counteract the pro-aggregating effects of MMP-2 by inhibiting platelet aggregation (Fernandez-Patron et al. 1999a). However, TCIPA was inhibited by phenanthroline that inhibits both MMP-2 and MMP-9 activities (Fernandez-Patron et al. 1999a). Therefore, it is likely that MMP-2 is the dominant platelet-regulating gelatinase under these conditions.

The MMP-2-dependant pathway of aggregation triggered by HT-1080 cells interacts with thromboxane and ADP-mediated pathways as revealed by experiments using selective inhibitors of these pathways of platelet aggregation. Thus, similar to agonist induced platelet aggregation (Sawicki et al. 1997) the major pathways of aggregation interact to stimulate TCIPA.

It has previously been shown that platelet MMP-2 is translocated during agonist induced aggregation to the platelet surface membrane and proposed that the reactions of MMP-2 with platelet integrin receptors such as GP Ib and GP IIb/IIIa mediate aggregation (Sawicki et al. 1998, Martinez et al. 2001, Radomski et al. 2001). Moreover, aggregation induced by HT-1080 cells was inhibited by the antagonist of the fibrinogen receptor N-Acetyl-Pen-Arg-Gly-Asp-Cys indicating that the expression of $\alpha_{IIb}\beta_3$ integrin receptor is a common pathway mediating TCIPA. Furthermore, Brooks et al. have recently shown that MMP-2 is localized to the surface of invasive cells with the integrin $\alpha_v\beta_3$ thereby facilitating

directed cellular invasion (Brooks et al. 1996). Therefore, it is likely that the interactions between platelet and cancer cell surface integrins and MMP-2 are important in mediating the aggregating effects of this MMP during TCIPA.

In addition to degradation of the cellular basement membrane and stimulation of TCIPA, MMP-2 also exerts some vascular effects that could contribute to carcinogenesis. Recently, it has been shown that MMP-2 cleaves big endothelin-1 to yield a novel vasoactive peptide medium endothelin-1 (Fernandez-Patron et al. 1999b). Endothelin-1 is a vasoconstrictor peptide that is known to affect tumor cell transduction mechanisms and stimulate tumor growth (Bagnato et al. 1997, Asham et al. 1998, Shankar et al. 1998, Moraitis et al. 1999).

NO in Cancer

The role of NO in cancer growth, invasion, and metastasis has been extensively studied. However, there has been considerable controversy in the literature regarding whether NO promotes or inhibits cancer growth, invasion, and metastasis. This is not entirely surprising considering the complex and multifaceted actions of NO including regulation of vasodilatation (Palmer et al. 1987), cell adhesion (Radomski et al. 1987b, Kubes et al. 1991), and its effects on cellular growth, proliferation, and cell migration (Lepoivre et al. 1991, Schini-Kerth et al. 1999, Goligorsky et al. 1999). Some studies have demonstrated that NO may promote tumor growth. Mortensen et al. have shown that inhibition of MCF-7 eNOS activity resulted in cancer cell apoptosis (Mortensen et al. 1999b). Furthermore, the NOS substrate L-arginine stimulated in vitro bladder carcinoma

growth in a dose-dependent manner, an effect reduced by NOS inhibition (Morcos et al. 1999). Moreover, NO may stimulate tumor growth by causing increased tumor neovascularization and blood flow. Indeed, four recent studies report that NO plays an important role in mediating tumor-induced angiogenesis (Jenkins et al. 1995, Bauer et al. 2000, Eroglu et al. 1999, Jadeski et al.1999). However, other researchers found that NO can decrease the rate of carcinogenesis. Indeed, high levels of eNOS in microvessels around breast cancers were associated with increased patient survival (Mortensen et al. 1999a). Furthermore, low amounts of NO donors when combined with inhibitors of farnesyltransferase exert a potent apoptosis-promoting effect in a human breast cancer cell line (Pervin et al. 2001). Moreover, other studies have shown that human colon carcinoma cells isolated from metastases exhibited lower NO activity than cells isolated from the primary tumor, and the metastatic cells were more potent inducers of platelet aggregation (Radomski et al. 1991). In addition, it has been established that an inverse correlation exists between the expression of endogenous inducible nitric oxide synthase (iNOS) and NO production by metastatic cells and their metastatic potential (Xie and Fidler 1998, Dong et al. 1994, Tschuggel et al. 1999a, Tschuggel et al. 1999b). Finally, it has been shown that induction of iNOS expression by cytokines such as tumor necrosis factor-α and interferon-β induced tumor apoptosis and inhibited tumor growth (Binder et al. 1999, Xu et al. 1998). Interestingly, Ambs et al. have shown that induction of iNOS in tumor cells with wild type p53 genes resulted in inhibition of tumor growth, but iNOS expression in tumor cells with mutant p53 resulted in increased

tumor growth, VEGF expression, and neovascularization of the tumor (Ambs et al. 1998). Thus, NO may either promote tumor growth or be tumoricidal depending on a number of factors such as the differentiation state of the tumor, its genetic status, vascularization, activation of tumor adhesion receptors, and the concentrations of NO in the tumor microenvironment.

In the experiments described in this thesis, the NO donors, SNAP and GSNO, inhibited platelet aggregation induced by HT-1080 cells in a concentration-dependent manner. Furthermore, these compounds inhibited the release of MMP-2 during TCIPA. Thus, this ability of NO to inhibit MMP-2 release shows that there is a "cross talk" between NO and MMP-2 during TCIPA. Interestingly, SNAP and GSNO also inhibited the release of MMP-2 from HT-1080 cells.

To determine whether the effects of NO on TCIPA were mediated by cyclic GMP or not, ODQ a selective inhibitor of the soluble guanylyl cyclase was used (Moro et al. 1996). ODQ was able to reverse the inhibition of TCIPA by the NO donors SNAP and GSNO in a concentration-dependent manner. These results demonstrate that inhibition of TCIPA by NO is cyclic GMP-dependant. In addition, ODQ was able to abolish the inhibition of MMP-2 release from platelets by SNAP and GSNO during TCIPA. Thus, both inhibition of TCIPA and the release of MMP-2 are controlled by cyclic GMP.

In addition, total NOS activity of the A549 and HT-1080 cell lines was determined. The NOS activity of the A549 cell line was found to be greater than that of the HT-1080 cells. Thus, this may further explain why HT-1080 cells are

more potent to induce platelet aggregation when compared to A549 cells. Indeed, higher generation of NO by the A549 cells could represent a mechanism by which these cells reduce their own release and actions of MMP-2. These results are consistent with those obtained by Radomski et al. (Radomski et al. 1991).

In conclusion, the first part of my studies has shown that aggregation of platelets by HT-1080 fibrosarcoma cells depends, in part, on the release of MMP-2 from platelets and cancer cells. The activation of TCIPA by MMP-2 is regulated by NO and cyclic GMP.

VWF and Glycoprotein Receptor Regulation during TCIPA

In the second part of my investigation I studied TCIPA in the presence of sVWF, a novel solid-phase agonist (Stewart et al. 1997). As vWF is a major adhesion protein contributing to the interactions between platelets and the vascular wall (Ruggeri et al., 1999), the use of sVWF added yet another dimension to studies on TCIPA that have considered until now only the platelet-tumour cell relationship (Honn et al. 1981; Radomski et al. 1991, Jurasz et al. 2001). In addition, the effects of NO and PGI_2, two known inhibitors of platelet aggregation (Radomski et al. 1987c and 1987d; Moncada et al. 1976) and TCIPA (Radomski et al. 1991; Jurasz et al. 2001; Honn et al. 1981; Menter et al. 1984) were investigated on TCIPA-sVWF-induced platelet activation.

As reported in the first part of this thesis human fibrosarcoma cells HT-1080 caused TCIPA. Preincubation of platelet samples with sVWF resulted in potentiation of TCIPA. This reaction consisted of two phases: the first dependent

upon platelet adhesion to sVWF, and the second characterized by formation of tight sVWF-platelet-tumor cell aggregates. These data show that vWF appears to play a crucial role in the interactions between cancer cells and platelets *in vitro*, and most likely *in vivo*. In this context, it is interesting to note that numerous studies have shown that cancer patients have elevated levels of plasma vWF. These include laryngeal (Paczuski et al. 1999), renal (Oleksowicz et al. 1999), colorectal (van Duijnhoven et al. 1993), cervical (Gadducci et al. 1993), prostate (Ablin et al. 1988), and head and neck cancers (Sweeney et al. 1990). Moreover, Oleksowicz et al. (1999) have shown that patients with metastatic disease have elevated levels of highly polymeric forms of vWF due to a deficiency of vWF-processing protease. Furthermore, increased thrombotic readiness detected in cancer patients may be due to the elevated plasma levels of vWF (Green et al. 1997). In addition, patients with malignant breast cancer have increased vWF content in the cytosol of the malignant tissue (Pratt et al. 1989). Importantly, it has been demonstrated that the staging and tumour size correlate with disease progression whereby patients with higher plasma vWF have more advanced disease (Paczuski et al. 1999; Gadducci et al. 1993; Pratt et al. 1989). Experimentally, co-culture of human HRT-18 colon cancer cells with human umbilical vein endothelial cells leads to an increase in vWF release from the endothelial cells and this may lead to enhanced platelet adhesion (Morganti et al. 1996). Finally, platelet aggregation induced by human MCF breast cancer cells is increased in cancer patients with elevated levels of vWF (Oleksowicz et al. 1999). The mechanisms that lead to an increase in plasma vWF in cancer

patients remain unclear; however, they may include damage to the endothelium, platelet activation and aggregation or tumour angiogenesis. Whatever the mechanisms that underlie increased release of vWF may be, clinical and experimental evidence strongly indicate that this protein plays an integral role in haematogenous spread of cancer.

Therefore, I have also studied the mechanisms of the activator effects of sVWF on TCIPA focusing upon two major platelet receptors GP Ib and GP IIb/IIIa.

In agreement with our previous work (Radomski et al. 2001), preincubation of platelets with sVWF alone caused increased surface expression of GP Ib. This enhanced expression of GP Ib is likely to support platelet adhesion to this ligand (Radomski et al. 2001) in light of GP Ibα/VWF-mediated tethering (Ruggeri 1999). In contrast, TCIPA resulted in a significant reduction of GP Ib expression on platelet surface both in the presence or absence of sVWF. Decreased surface expression of GP Ib has been already reported as a result of thrombin-induced platelet aggregation or the exposure of platelets to a foreign surface (Keh et al. 1996; Michelson et al. 1996; Mellgren et al. 1995). This reduction could be a result of receptor internalisation or proteolysis and shedding (Mellgren et al. 1995, Hughes et al. 2000; Bergmeier et al. 2000; Kinlough-Rathbone et al. 2000). The biological significance of reduced expression of GPIb during aggregatory reactions is unclear.

In contrast to GP Ib, TCIPA-sVWF-mediated platelet activation was clearly associated with increased surface expression of GP IIb/IIIa emphasizing, once

again, an important role of this receptor in platelet activation by tumour cells (Jurasz et al. 2001a).

Platelet activation induced by tumor cells in the presence of sVWF was inhibited by NO and prostacyclin highlighting further the regulatory role of these mediators in the interactions between platelets, the vessel wall and tumor cells. We and others have previously shown that these inhibitors down-regulate TCIPA (Honn et al. 1981; Radomski et al. 1991; Jurasz et al. 2001a). Since the regulatory effects of NO and PGI_2 on agonist-induced platelet aggregation are dependent on modulation of platelet receptor glycoprotein function, the effects of these inhibitors on TCIPA-sVWF-stimulated changes in GP Ib and GP IIb/IIIa expression were investigated. PGI_2 inhibited the surface expression of GP IIb/IIIa, while GSNO did not. Since both inhibitors reduced TCIPA-sVWF-stimulated platelet activation, differential effects of NO and PGI_2 on GP IIb/IIIa expression were surprising. Therefore, investigations of whether GSNO had any effects on the activation of GP IIb/IIIa using the PAC-1 antibody, which recognizes the activated form of GP IIb/IIIa were carried out. The study revealed that NO inhibited the activation of GP IIb/IIIa caused by cancer cells. Recently, Keh and colleagues (1996) have shown that GSNO inhibits both the increased surface expression and activation of GP IIb/IIIa in platelets stimulated by thrombin. Our own data show that GSNO only inhibited GP IIb/IIIa activation. This discrepancy maybe due to procedures used for sample preparation and/or the nature of the platelet activating agents. To measure total GP IIb/IIIa by flow cytometry Keh and colleagues fixed their platelet samples and stored them at

4°C for up to 4 hours. Measurement of activated GP IIb/IIIa with the PAC-1 antibody, in our experiments, was performed on platelet samples that were not fixed. Fixation of platelets may alter platelet surface receptor expression and/or impair fluorescence intensity. The timing of flow cytometry assays of non-fixed samples is crucial; therefore, all our measurements were performed precisely at 5 minutes after sample preparation. Furthermore, the discrepancy in results may arise from the differences between platelet activation by thrombin versus HT-1080 fibrosarcoma cells. Indeed, the first part of my studies has shown that platelet activation by HT-1080 cells is a complex process that involves the activation of the ADP, thromboxane, and MMP-2 pathways of platelet aggregation (Jurasz et al. 2001). Activation and aggregation of platelets by cancer cells is not only a complex process, but also a very potent one. Indeed, 10^3 cancer cells could induce aggregation of 10^8 platelets. Thus, NO may not be able to inhibit up-regulation of platelet surface GP IIb/IIIa induced by HT-1080 cells, but it does inhibit the conformational change in GP IIb/IIIa that precedes platelet aggregation. In contrast to NO, PGI_2 down-regulated an increase in GP IIb/IIIa surface expression induced by TCIPA-sVWF. Interestingly, it has been shown that tyrosine phosphorylation of platelet proteins associated with aggregation is inhibited by cAMP-, but not cGMP-elevating agents (Pumiglia et al. 1990). As tyrosine phosporylation is linked to GP IIb/IIIa function (Golden et al. 1990; Parise et al. 1990), a differential regulation of function of this receptor by NO and PGI_2 may be explained by the differences in signal transduction mechanisms operated by the PGI_2-cAMP and NO-cGMP systems.

Neither PGI_2 nor NO exerted any significant effects on reduction of GPIb surface expression caused by TCIPA-sVWF. This data indicates that inhibition of TCIPA-sVWF by NO and PGI_2 is related to their interactions with GP IIb/IIIa rather than GP Ib.

Platelet activation leads to the formation of platelet particles of various sizes. Therefore, the flow cytometry assay performed has analysed the effects of stimulators of aggregation (HT-1080 cells and sVWF) and inhibitors (PGI_2 and GSNO) on large, medium, and small sized platelet populations. It was found that these activators and inhibitors affected different sized platelet populations in the same manner.

This model of TCIPA including sVWF may be of use to further understand the process of haematogenous metastasis of cancer. It is likely that a haematogenous metastasising cancer cell should follow the path of least resistance (Cotmore and Carter 1973). This means that the cell will arrest and extravasate at a site in the vasculature where vascular integrity has been disrupted. This site of vascular injury will undoubtedly already have platelets adhering and aggregating to it in an effort to control bleeding and to repair the damaged vessel. Therefore, our data suggests that the ability of the tumor cell to aggregate platelets and consequently up regulate platelet surface GP IIb/IIIa gives the tumor cell-platelet aggregate the advantage of engaging platelets already in the process of aggregation at the site of vascular injury.

In conclusion, the second part of my studies demonstrated that sVWF amplifies TCIPA. The effects of sVWF are largely mediated via increased

expression of GP IIb/IIIa in platelets. Finally, these actions of sVWF are modulated by NO and prostacyclin.

TIMP-4 in TCIPA

Finally, the last phase of my investigations involved the study of the role of endogenous inhibitors of matrix metalloproteinases in TCIPA. Since TIMP-4 was identified to be the major TIMP present in human platelets, I examined its role in tumor cell-induced platelet aggregation. Human platelets incubated in the aggregometer released low basal levels of TIMP-4. These levels did not increase upon platelet aggregation by HT-1080 cells that also produce and release TIMP-4. This is in marked contrast to collagen induced platelet aggregation that results in the release of TIMP-4 during platelet aggregation. Furthermore, human recombinant TIMP-4 was shown to have a bi-phasic effect in inhibiting TCIPA that depended on the TIMP-4 concentration used. At 50 ng/ml TIMP-4 showed inhibitory activity; however, at higher concentrations it exerted no significant effect on TCIPA. The exact role TIMP-4 plays in platelets is not yet known; however, this data does support the hypothesis that TIMP-4 plays a role in inhibiting the activation of MMP-2, unlike TIMP-2 that is involved in the activation of this protease (Biggs et al. 2001). Under physiological conditions the release of TIMP-4 during platelet aggregation could serve to limit the formation of platelet aggregates and consequently pathological thrombosis. It is conceivable that TIMP-4 acts as a natural inhibitor of platelet aggregation *in vivo* much like NO produced during platelet aggregation to stop the uncontrolled proliferation of aggregates. The fact that the levels of TIMP-4 were not elevated during TCIPA

implies that HT-1080 cells may sequester the release of TIMP-4 from platelets. This ability of the tumor cells may provide them with a survival advantage by furthering their ability to aggregate platelets. Why recombinant TIMP-4 has a bi-phasic effect on TCIPA is unknown. However, it is known that TIMPs have other effects in addition to inhibiting MMPs. It has been shown that TIMPs may act as growth factors (Barasch et al. 1999) therefore it is conceivable that the MMP-2 inhibitory effects of TIMP-4 are opposed by some other properties of this inhibitor. Thus, the balance between MMPs and TIMPs plays a vital role in the determining the physiological and pathological actions of these molecules.

If TIMP-4 is the major TIMP expressed by platelets and it serves in inhibiting MMP-2 then the question remains of how MMP-2 is activated in or by platelets. To answer this question detailed studies on platelet membrane-type MMPs will have to be carried out along with the role of integrins such as $\alpha_v\beta_3$, which have been shown to play a role in the control of MMP-2 activity (Brooks et al. 1996).

Pharmacological Significance

The results shown in this thesis have clinical implications, as they show that NO donors not only inhibit TCIPA, but also reduce the release of MMPs from invasive tumor cells. Moreover, some SNAP derivatives such as glucose-2-SNAP and fructose-2-SNAP are also cytotoxic to cancerous cells (Hou et al. 1999, Cantuaria et al. 1999).

It is quite possible that the use of NO as a therapeutic tool in inhibiting TCIPA and thereby impeding the metastatic process would require platelet-

specific NO donors. In fact, platelet-non-specific NO donors such as organic nitrates (Mehta 1995), could potentially promote carcinogenesis by causing vasodilatation and increasing blood flow to a growing tumor. Interestingly, GSNO has been shown to be a relatively platelet-specific NO donor, as it inhibits platelet aggregation at concentrations that do not cause significant vasodilatation (de Belder et al. 1994). The mechanism(s) of these effects of GSNO are relatively platelet-specific requiring extracellular enzymatic metabolism of the GSNO molecule (de Belder et al. 1994, Gordge et al. 1998).

As of yet no MMP inhibitor trial has succeeded in the clinic. While there have been many compounds that have shown strong MMP inhibitory effects *in vitro* or in animal models, most MMP inhibitors have failed to reach the clinic due to failure of safety studies, or if they have reached the clinic they failed due to poor bioavailability and rapid metabolism (Greenwald 1999). Of the few MMP inhibitors that have reached the clinic and managed to have acceptable safety profiles and good bioavailablity, all have shown not to be efficacious in inhibiting the course of cancer disease. Some of the reasons for the failure of these compounds as discussed by Zucker and colleagues include: the exact role of MMPs in cancer is not fully understood, the use of non-specific inhibitors of MMPs, and finally the clinical trials were performed in the incorrect patient populations (Zucker et al. 2000). Zucker and colleagues argue that the precise role of MMPs in cancer is more complicated than had been anticipated. Many of the MMPs, which have been shown to promote tumor cell invasion, have also been reported to have anti-cancer effects due to the production of angiogenesis

inhibitors. The angiogenesis inhibitor angiostatin has been shown to be produced by MMP-2, MMP-3, MMP-7, MMP-9, and MMP-12 (O'Reilly et al. 1999, Lijnen et al. 1998, Patterson and Sang 1997, Dong et al. 1997). Inhibition of a wide spectrum of MMPs may result in inhibiting the beneficial role MMPs may play in cancer. Thus, the use of non-specific MMP inhibitors may also inhibit the anti-cancer effects of MMPs. In the future the use of specific MMP inhibitors may also not be the answer. No one MMP has been identified as the culprit that mediates tumor invasion and metastasis; rather, many MMPs have been shown to contribute to tumor invasion and metastasis. Inhibiting one will likely not result in the arrest of tumor invasion and metastasis. Finally, the most important reason of why MMP inhibitors have failed in clinical trials to date is the fact that all these trials took place in patient populations with advanced cancer where tumor invasion had already taken place and metastasis already established. MMP inhibitors have no cytotoxic effects against the tumor, thus once a metastasis has been established they should be anticipated not to have a protective effect. Clinical trials with patients in earlier stages of cancerous disease should be carried out to test the effect in patients with the most to gain from this class of drugs. Whether or not these suggested trials will be carried out or if the pharmaceutical industry has been discouraged from using this class of drugs by the current failures remains to be seen.

Furthermore, it remains to been seen if MMPs are the only means for tumor invasion and metastasis or if other mediators are involved in what looks like a more complicated process then first anticipated.

Perhaps a synergy between NO donors and MMP inhibitors may have a role in inhibiting metastatic disease. Evidence in support of NO controlling the release of MMP-2, not only from platelets, comes from the ability of SNAP and GSNO to inhibit the release of MMP-2 directly from the HT 1080 tumor cells. This result has clinical implications in that not only can NO donors potentially inhibit tumor cell-induced platelet aggregation, but may also have a therapeutic benefit in the treatment of metastatic cancer by directly inhibiting the release of matrix metalloproteinases from invasive tumor cells.

Furthermore, the fact that ODQ up regulated MMP-2 release during TCIPA further supports the idea that there is cross talk between the MMP and NO bioregulatory pathways and that NO controls the release of MMP-2. This interaction between the two pathways takes place downstream of the GC-S and the inhibition in MMP-2 release by NO occurs via a cGMP-dependant mechanism.

Moreover, the use of NO donors with MMP inhibitors may have synergistic effects in reducing TCIPA, and the release of MMPs from cancer cells. The synergistic effects of NO and MMP inhibition have already been demonstrated by Martinez and colleagues to inhibit the adhesion of platelets to fibrinogen (Martinez et al. 2001). Thus, the use of NO donors and MMP inhibitors together in the clinic may have synergistic effects in controlling TCIPA and metastasis.

Due to metastasis, cancer is a group of diseases that may have systemic affects. Since more than one region of the body may be affected many different cell types play roles in the progression and inhibition of the course of this

disease. Not only are the cancerous cells themselves involved but also other cells such as stromal cells, immune cells, endothelial cells, and platelets. In addition, non-cellular components of the body are also involved in cancer such as the extracellular matrix, the scaffolding that holds the cells together. Because of the often involvement of distant regions of the body in this disease more research should focus on how factors such as different cell types, the extracellular matrix, endogenous molecules that affect carcinogenesis, anatomical considerations, and cellular changes combine to affect the natural causes of cancer. Thus, I believe that treatment strategies should not only focus on the cancer cells themselves, but also expand to encompass and combine strategies that take into consideration the interactions of cancer cells with these factors.

Future Directions

Role of Platelets in Angiogenesis

I have already outlined the importance of sustained angiogenesis in carcinogenesis (Tumor cell, p. 33) The presence of both angiogenic promoters and inhibitors, such as VEGF and thrombospondin (Bornstein 2001), in platelets provides the rationale that platelets may play an important role in angiogenesis (Figure 44).

Angiogenesis Promoters	Angiogenesis Inhibitors
Vascular Endothelial Growth Factor*	Interferons α/β
Acidic/basic Fibrolast Growth Factor	Thrombospondin*
$\alpha_v\beta_3$ integrins*	Platelet activating factor-4*
Matrix metalloproteinases*	16KDa prolactin
	Angiostatin
	Endostatin
	PEX

(* indicates platelet derived factors)

Figure 44. Major angiogenesis-regulating factors.

One of the angiogenesis-regulatory factors is angiostatin (Figure 45). The idea that platelets may produce angiostatin was born from two lines of thought. First, platelets are known to convert plasminogen into plasmin on their surface (Deguchi et al. 1985). Second, platelets are known to contain on their surface the enzymes, such as MMP-2, that could potentially convert plasminogen to angiostatin (Sawicki et al. 1998). Moreover, MMP-2 has been shown to be the enzyme responsible for the production of angiostatin in a Lewis lung carcinoma (LLC-LM) model of concomitant resistance (O'Reilly et al. 1999). In addition to cancer cells being able to synthesize angiostatin (O'Reilly et al. 1994, Westphal et al. 2000), it has been shown that macrophages have the capacity to

synthesize angiostatin (Dong et al. 1997, Falcone et al. 1998).

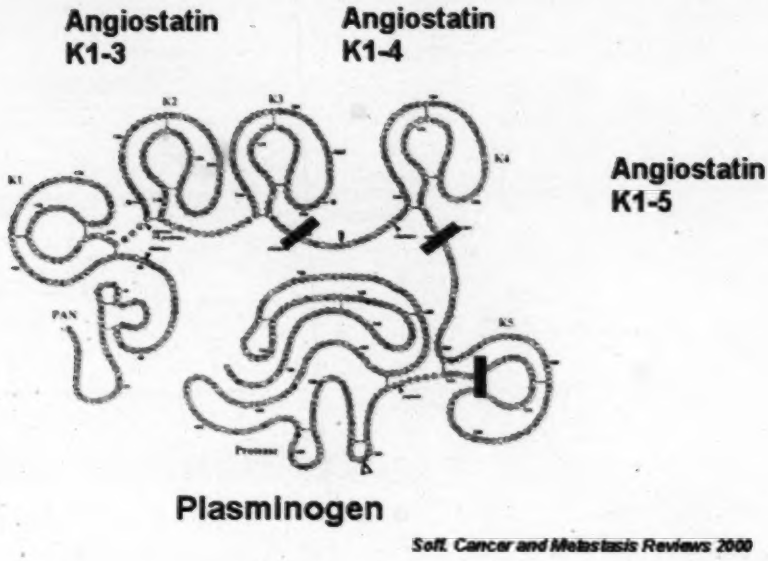

Figure 45. Proteolytic processing of plasminogen results in angiostatins of different size that are classified based on the number of kringles (K) present.

We have recently discovered the formation of the angiogenesis inhibitor angiostatin in human platelets. Angiostatin is a potent inhibitor of endothelial cell proliferation and tumor angiogenesis derived from the proteolytic processing of the plasminogen molecule (O'Reilly et al. 1994). Using immunoblotting we have detected the formation of an angiostatin corresponding to approximately 57 KDa in platelet pellets and in the pellets of platelets aggregated by HT-1080 fibrosarcoma cells. Also, using the same technique we have detected the formation of an angiostatin corresponding to approximately 50 KDa kringles in the releasates of platelets and platelets aggregated by HT-1080 cells (Figure 46).

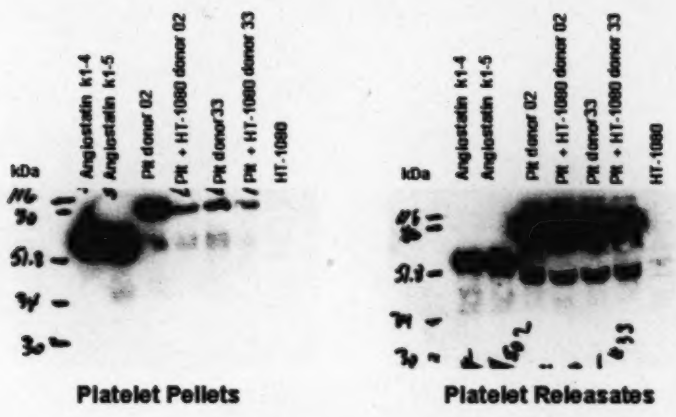

Figure 46. Detection of the angiogenesis inhibitor angiostatin in platelets and TCIPA.

The formation of angiostatin by HT-1080 cells was not detected indicating this to be a platelet specific event. To test if this truly is angiostatin being formed by platelets will require the isolation and purification of the proteins in question followed by their sequencing to confirm this result. In addition, functional tests would need to be carried out to test whether these proteins have properties similar or identical to angiostatin. This would require testing them in an angiogenesis assay.

ADAMs Family in TCIPA

A second line of investigation in the future should focus on the role of the ADAM family of disintegrin-like metalloproteinases in TCIPA. ADAMs stands for *a* *d*isintegrin *a*nd *m*etalloproteinase-containing protein. These proteinases share a high level of sequence homology and domain organization with snake venom metalloproteinases (SVMPs) or a group of proteases also known as adamlysins (Stone et al. 1999). Like the matrix metalloproteinases the ADAMs are a family of

zinc-dependent proteinases. They contain a pre- and pro-domain, a catalytic domain, a disintegrin-domain, a cys-rich domain, an EGF-domain, a transmembrane-domain, and a cytoplasmic tail. What is of particular relevance to TCIPA, and hemostasis in general, is the disintegrin-domain. As mentioned previously, many of today's GP IIb/IIIa receptor antagonists that are in clinical use for the treatment of acute coronary syndromes are based on the disintegrin sequence that is found in proteins in the venoms of viper snakes. The disintegrins found in the venom of viper snakes will bind to platelet GP IIb/IIIa and other integrins and result in hemorrhage and possibly death by the inhibition of platelet aggregation after a snake bite (Bjarnason and Fox 1994). The disintegrin sequence of ADAMs proteinases contains a RGE sequence that is slightly different than the RGD-recognition sequence required for integrin binding. This sequence is present in all known ADAMs except ADAM15, which contains the RGD sequence in its integrin-binding domain (Kratzschmar et al. 1996, Zhang et al. 1998). ADAMs have been found in many tissues including the liver, brain, and endothelium (Wolfsberg et al. 1995, Herren et al. 1997); whether they are found in platelets still requires investigation; however, it is not inconceivable. Thus, if and what role the ADAMs play in platelets, hemostasis, and TCIPA still needs to be investigated.

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